Vol. X, No. 2 June 1916

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

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LONDON: Fetter Lane, E.C.
EDINBURGH: 100, Princes Street

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H. K. LEWIS & Co., Ltd., 136, Gower Street, London, W.C.
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BOMBAY, CALCUTTA AND MADRAS: Macmillan and Co., Ltd.
TORONTO: J. M. Dent & Sons, Ltd.
TOKYO: The Maruzen-Kabushiki-Kaisha

1916

Price Seven Shillings net.

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XVII. ADSORPTIVE STRATIFICATION IN GELS.

By SAMUEL CLEMENT BRADFORD.

From the Science Museum.

(*Received March 15th, 1916.*)

In a previous paper [Bradford, 1916] the author proposed the theory that the Liesegang phenomenon might be due to gradual adsorption of the solute in the gel by the growing precipitate, so that, the adjacent layer of gel presently becoming exhausted of solute, the reacting substance would be able to diffuse further into the gel before commencing to form a fresh stratum of precipitate. Hatschek [1914] had proved that Ostwald's hypothesis of metastable solutions was not of general application. Moreover experiments with gels containing additional quantities of the soluble products of the double decomposition, which had been assumed by some to play an essential part in the formation of the strata, showed no appreciable effect on their character or separation. The theory affords an explanation of the fact observed by Pringsheim that diffusion takes place from the hypertonic into the hypotonic region, whether liquid or gel, and that, as recorded by the author, in the absence of a precipitate, mutual interdiffusion results. By working with a coloured solute, such as potassium chromate, in the gel, the gradual exhaustion of the zone near the precipitate could be observed. This is shown in Fig. 1 (Plate I) which represents a tube prepared with N/20 K_2CrO_4 in 1 % agar on which a N/5 solution of $Pb(NO_3)_2$ was poured. Stratification became more evident lower down the tube, but the yellow colour was then less marked. Experiments in 1 % agar in which columns of fuller's earth were embedded above dye solutions showed similar adsorption, though not so intense, since the surface was not being constantly renewed by precipitation.

The agar gel is easily prepared by boiling 10 g. of agar sticks in a litre of water and straining through muslin while hot into a wide-mouthed bottle. The resulting gel should be perfectly clear, otherwise stratification may be hindered, or altogether prevented. When required for use a quantity of the

jelly is warmed in a beaker on the water-bath and strained into stoppered test-tubes graduated to contain 15 and 25 cc. The tubes are previously prepared by adding the requisite quantity of standard solutions of the reagents intended, so that, when filled to the 15 cc. mark with agar, the gel will be of the desired concentration. When the gel is set, standard solutions are poured above up to the 25 cc. mark. The concentrations which frequently give the best results are about N/20 for the gel and N to N/5 for the solution above.

Experiments have been made with a variety of insoluble substances. Potassium iodide treated with lead nitrate, and manganese sulphate with liver of sulphur, are noticeable as rapidly producing clearly defined strata. The structure of the manganese sulphide in Fig. 2 (Plate I) closely resembles that of certain limestone formations from Fulwell Hill district. Precipitates which refuse to stratify when prepared in straight tubes, generally yield the desired result when the reacting solutions are poured into the two limbs of a U-tube half filled with agar gel. Copper carbonate and ferrocyanide, cobalt ferricyanide and barium sulphate are examples. As the concentration of the gel falls slowly in U-tube experiments, owing to the reserve of the more dilute reagent in solution above the gel, the discs of precipitate are formed at nearly equal intervals after the first two or three. A necessary condition for the production of clearly defined strata appears to be that the reacting solutions should not be too strong. From his experiments on the adsorption of soluble substances by filter paper Evans [1906] showed that the proportion of solute removed increases with dilution, but that, in the cases examined, no adsorption could be observed in strong solution. After diffusing through a column of gel in a U-tube, the concentrations of the meeting solutions are reduced to the necessary dilution, while sufficient quantities of the reagents are available to produce well defined discs. So far as has been observed, under these conditions stratification is well developed.

The distances between the layers should depend mainly upon C, the concentration of the gel, and the ratio U/C, U being the concentration at the boundary surface between the solid and gel phases at the temperature of the experiment. The distances should therefore be characteristic of the solute in the gel. They should also be affected by the permeability of the precipitate to the reagent diffusing through, and, to some extent, by the rate of diffusion of the reagent in the gel. If the adsorption of one solute is affected by the presence of a considerable quantity of another, as seems to be the case, stratification would also be influenced by increasing the concentration of the

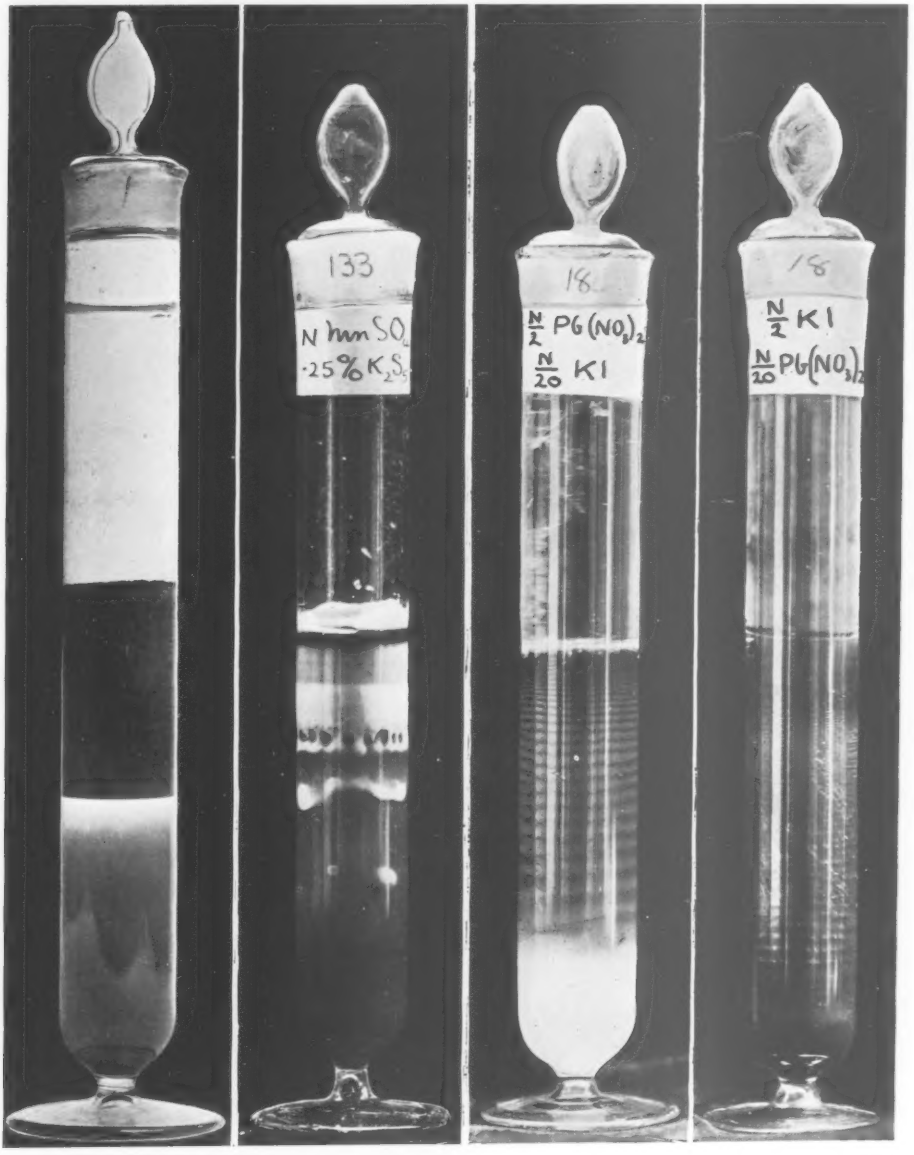


Fig. 1 Fig. 2 Fig. 3 *a* Fig. 3 *b*

reagent above the gel. In the case of a pair of reagents with different ratios U/C, the distances apart of the strata should turn upon which of the reagents is dissolved in the gel. This is illustrated by the tubes represented in Figs. 3 *a* and 3 *b* (Plate I). The former contained N/20 KI in 1 % agar with N/2 $Pb(NO_3)_2$ solution above, while in the latter tube the gel was made up with N/20 $Pb(NO_3)_2$ and treated with N/2 solution of KI.

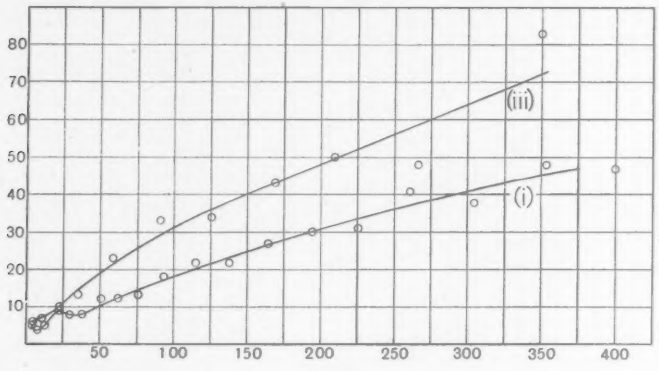


Fig. 5

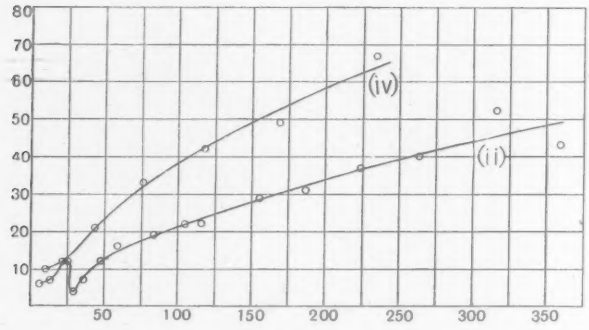


Fig. 6

Experiments with tubes, Fig. 4 (Plate II), containing

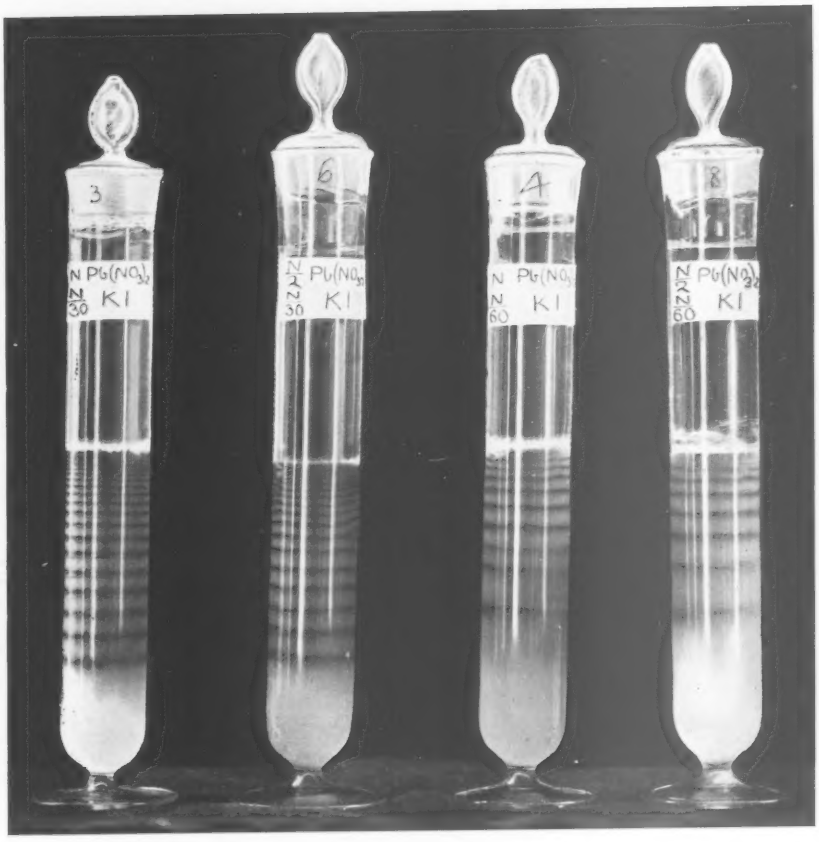
(i) N/30 KI agar + N $Pb(NO_3)_2$, (ii) N/30 KI agar + N/2 $Pb(NO_3)_2$, (iii) N/60 KI agar + N $Pb(NO_3)_2$ and (iv) N/60 KI agar + N/2 $Pb(NO_3)_2$ show how, in this case, the distances apart of the layers are dependent rather upon the concentration of the solute in the gel than upon that of the reagent above. The curves, Figs. 5 and 6, were drawn freely through

points obtained by plotting the distances apart of the strata as ordinates against the distances down the tube as abscissae. The lower curves, which correspond to nos. (i) and (ii), are too nearly coincident to be drawn on the same diagram. There is a little more distinction between the upper curves. As the strata take some days to form, the irregularities of the points may be due to variations of temperature. In many cases, however, the formation of the laminae is more regular.

Since Lagergren [1899] had observed a negative adsorption in the solutions of certain salts, *e.g.* sodium chloride, it seemed desirable to ascertain whether stratification could be obtained in gels impregnated with such a substance. Accordingly gels were prepared containing sodium chloride of various concentrations, and upon these were poured solutions of silver nitrate. The character of precipitate produced was quite different from that resulting with other solutes, being merely a diffuse cloudiness without any definite margin indicating the limit of the diffusion, which also took place much more quickly than had hitherto been observed. In no instance was any stratification noticed. However tubes prepared with silver nitrate of similar concentrations in the gel, and sodium chloride above, also showed no layers. This precipitate however was bounded by a definite margin, as in other cases, until the gel had become nearly exhausted of solute, when a diffuse precipitate rapidly extended to the bottoms of the tubes.

The differences in the character of the AgCl precipitate, according to which reagent is contained in the gel, recall similar divergencies well-known in volumetric analysis when decinormal solutions of silver nitrate and sodium chloride are mixed. It may be that the two varieties of milky and curdy precipitates are due to their formation in solutions having, in the one case negative, and in the other, positive adsorption values, which would affect the concentrations of the solutions in the neighbourhood of the precipitates. This would seem to be supported by the fact that many metallic chlorides give a curdy precipitate when silver nitrate is added to them in dilute solution.

Since gels containing sodium chloride could not be made to produce stratification in straight tubes, experiments were made in U-tubes containing pure 1 % agar, the limbs of which were filled up with solutions of sodium chloride and silver nitrate respectively. Contrary to expectation, stratification was produced whichever reagent was hypertonic, although the same differences in the character of the precipitates were noticed. When hypertonic sodium chloride diffused into silver nitrate a dense curdy column of silver chloride was produced, separated, at intervals of about a centimetre, by clear cut



i ii iii iv

Fig. 4

interspaces about a millimetre thick of transparent gel. Under the reverse condition only a slight diffuse cloudiness was formed on the first meeting of the solutions. This extended along the tube for about 12 or 15 mm., gradually filling the area of the tube and becoming a little denser. As further diffusion took place the first very light precipitate gradually disappeared, as if it had been swept up towards the denser portion by the diffusing reagent. This happened in every case, and, as other instances of the motion of visible particles in the gel have been observed, the effect may be due to this cause. After a time thin discs of denser precipitate appeared to be forming in the diffuse column. This was attributed to the backward diffusion of the sodium chloride after the strength of the silver solution had been diminished both by precipitation and adsorption. Obviously this explanation was not of general application. Consequently U-tubes of agar gel were treated with N/10 $AgNO_3$ and N/100 NaCl. After the production of the first light streaks, a great number of thin discs began to make their appearance at about 4 mm. apart, the distance rapidly decreasing to about 1 mm. On account of the low concentration of sodium chloride employed the discs could not be explained by alternate diffusion of the reagents. It seemed to follow, either that sodium chloride is positively adsorbed by silver chloride, or that the lamination resulted from the action of negative adsorption.

Attempts were then made to determine the nature of the adsorption of sodium chloride from solution by precipitated silver chloride. 100 cc. of decinormal NaCl solution were poured into well-stoppered bottles and to these were added 50 cc. of decinormal silver nitrate, thus producing a milky precipitate of silver chloride in a N/30 solution of sodium chloride. The bottles were allowed to stand 48 hours with occasional shaking, the clear supernatant liquid was then drawn off, 50 cc. at a time, in a pipette provided with a stopcock to prevent agitation of the precipitate. Titrations with decinormal silver nitrate indicated an adsorption of – 0·06 millimols per gram of AgCl in thirtieth normal solution, and of – 0·04 millimols in 0·0094 normal solution at 14°[1]. Lagergren's figures for charcoal in 0·98 normal concentration were – 0·09 millimols per gram at 0°. These experiments indicated that the stratification in sodium chloride gels must be explained by negative adsorption.

Further observations with U-tubes showed the occurrence of three types

[1] 50 cc. supernatant liquid required 16·9, 16·9, 16·85, 4·7, 4·7 and 4·8 cc. N/10 $AgNO_3$ respectively.

of stratification: (i) separate discs with no remaining diffuse precipitate, as previously described in the case of N/100 NaCl, (ii) a number of denser discs of chloride with extensions of very diffuse precipitate in the direction of diffusion and clear interspaces, and (iii) a column of diffuse precipitate interspersed, at intervals of about 2 mm., with very thin laminae of much denser precipitate. These effects may possibly be explained as follows. Owing to the negative adsorption of sodium chloride, the region of gel near the precipitate becomes gradually weaker in solute, while the next layer increases in strength. The excess of sodium chloride in this zone will tend to diffuse away, but will be hindered by the momentum of the particles flowing down the tube towards the precipitate. If the gel is very dilute, the region next the precipitate may not retain sufficient solute to produce a precipitate and the silver salt will be able to diffuse further until it enters the zone of stronger sodium chloride. This would give rise to a stratification of the first type. A gel which had been treated with a somewhat more concentrated solute might yield a precipitate gradually becoming less dense to zero before the diffusing silver salt reached the stronger layer of sodium chloride. This would correspond to type two. If the sodium chloride were again a little stronger, the clear spaces might not be produced at all, as in the third type.

Experiments in hand, which have now been interrupted, indicate that the remarkable influences of different gels upon the results obtained may be explained by v. Weimarn's theory on the assumption of protective action. For example lead iodide gives stratifications of gelatinous precipitate with difficulty in 5 % gelatin, while coarse precipitates in beautiful laminae occur in 1 % agar, and merely large aggregates in silicic acid gels. These results can be modified by varying the solubility of the precipitate, the concentration of the solute, or in other ways.

The following table (p. 175) records the positions of the strata formed in a number of experiments, measured in millimetres from the top of the gel. In many cases there were previous strata which were too close together for their positions to be read on a scale. The gel was 1 % agar except in the first case and the substance first mentioned was dissolved in the gel.

REFERENCES.

Bradford (1916). *Science Prog.* **10**, 369.
Evans (1906). *J. Phys. Chem.* **10**, 290.
Hatschek (1914). *Kolloid Zeitsch.* **14**, 115.
Lagergren (1899). *K. Svenska Vet. Akad. Handl.* **24**, 2, Nos. 4 and 5.

Reference No.																								
9*a*	Silver dichromate in 10 % gelatin	3·6	7·6	11·75	16·2	21·2	26·25	32·1	38·1	44·25	53·1													
13	N/30 $CaCl_2$ + conc. Na_2CO_3	26·0	29·0	34·5	43·0	54·0																		
14	N/20 $CaCl_2$ + conc. Na_2CO_3	22·0	23·5	26·75	30·0																			
19	N/20 Na_2CO_3 + dil. $CaCl_2$	20·5	22·5	25·0	28·0	32·0	35·5																	
20	N/20 Na_2CO_3 + dil. $CaCl_2$	20·5	22·5	25·5	28·5	32·5	36·0																	
21	N/20 Na_2CO_3 / N/20 NaCl + dil. $CaCl_2$	20·5	22·5	24·5	—	33·0	35·5																	
22	N/20 Na_2CO_3 / N/20 NaCl + dil. $CaCl_2$	20·5	23·0	—	—	31·5	35·5																	
38	dil. $AgNO_3$ + conc. $K_2Cr_2O_7$	14·0	16·3	19·5	23·7	29·0	35·5	44·0																
50	N/20 Na_2CO_3 + conc. $CaCl_2$	24·5	28·5	32·5																				
51	N/20 Na_2CO_3 / N/20 NaCl + conc. $CaCl_2$	24·0	28·5	33·5	38·0																			
79	N/60 $K_2Cr_2O_7$ + N/5 $Pb(NO_3)_2$	56·1	57·2	58·5	59·8	61·0																		
119	N/50 KI + N $Pb(NO_3)_2$	0·4	1·0	1·9	2·9	4·0	5·2	6·5	8·1	9·9	12·0	14·5	17·1	20·2	23·4	27·0	30·9	35·0	39·4	44·1				
120	N/20 KI + N $Pb(NO_3)_2$	5·0	5·7	6·5	7·4	8·4	9·5	10·7	12·0	13·4	15·1	17·0	19·1	21·4	23·8	26·2	29·5	33·4	37·7	42·3				
129	N/50 KI + N/5 $Pb(NO_3)_2$	0·5	1·1	1·9	3·0	4·2	5·6	7·2	9·1	11·2	13·7	16·6	20·3	24·6	shorter tube									
139	N/50 KI + N$Pb(NO_3)_2$	0·6	1·3	2·3	3·4	4·8	6·2	7·8	9·4	11·2	13·6	15·7	18·3	21·8	25·2	28·8	32·5	36·5						
140	N/50 KI + N/2 Pb $(NO_3)_2$	1·3	2·4	3·7	5·0	6·8	8·8	11·2	13·5	16·3	19·7	23·0	26·7	30·8	35·0									
130	N/20 $MnSO_4$ + 5 % K_2S_x	2·7	3·8	4·5	5·7	6·8	7·9	9·1	10·1	11·3	13·0	14·3	15·9	16·8	17·9	18·9	20·0	23·0	27·0	32·4	40·3	51·2		
133	0·25 % K_2S_x + N $MnSO_4$	2·0	—	—	8·0	10·5	15·0	19·0	26·0	40·0														
134	0·09 % K_2S_x + N $MnSO_4$	7·0	11·0	16·0	24·0	33·0	45·0																	
154	N/30 KI + N $Pb(NO_3)_2$	0·6	1·1	1·6	2·4	3·2	3·9	5·0	6·2	7·5	9·3	10·8	12·8	15·0	17·1	20·3	23·6	26·8	30·5	34·8	39·7			
155	N/30 KI + N Pb $(NO_3)_2$	0·5	0·9	1·4	2·3	3·1	3·9	5·1	6·3	7·6	9·4	11·6	13·8	16·5	19·5	22·6	26·7	30·5	35·3	40·0				
156	N/60 KI + N Pb $(NO_3)_2$	0·6	1·3	2·3	3·6	5·9	9·2	12·6	16·9	21·9	26·7	35·0												
157	N/60 KI + N Pb $(NO_3)_2$	0·4	1·3	2·8	4·9	7·6	10·5	13·6	17·8	24·2	31·6													
158	N/30 KI + N/2 Pb $(NO_3)_2$	0·6	1·3	2·5	2·9	3·6	4·8	6·4	8·3	10·5	12·7	15·6	18·7	22·4	26·4	31·6	35·9							
159	N/30 KI + N/2 Pb $(NO_3)_2$	0·9	1·6	2·3	3·3	4·2	5·7	7·0																
160	N/60 KI + N/2 Pb $(NO_3)_2$	1·0	2·2	4·3	7·6	11·8	16·7	23·4																
161	N/60 KI + N/2 Pb $(NO_3)_2$	0·9	2·7	4·3	7·2	11·2	15·8	21·3																
184	N/20 KI + N/2 Pb $(NO_3)_2$	3·4	4·0	4·8	5·6	6·3	7·2	8·1	9·0	10·2	11·6	13·1	14·9	16·8	18·8	21·6	23·8	25·2	27·9	30·7	33·8	37·1	41·8	
183	N/20 Pb $(NO_3)_2$ + N/2 KI	18·4	18·9	19·5	20·1	20·7	21·3	22·0	22·7	23·4	24·1	24·8	25·5	26·3	27·2	28·1	29·1	30·2	31·3	32·4	33·5	34·7	35·8	
														37·1	38·4	39·6	40·9	42·4	44·1	45·9	48·0	50·1	52·0	

XVIII. A NEW COLOUR REACTION FOR "OXYCHOLESTEROL."

By MARY CHRISTINE ROSENHEIM.

From the Physiological Laboratory, King's College, London.

(*Received March 20th, 1916.*)

In a previous communication dealing with the presence of "Oxycholesterol" in brain [1914], I have mentioned that this substance, when moistened with dimethyl sulphate (techn.) gives immediately a characteristic purple colour reaction, while under the same conditions cholesterol remains unaffected[1]. The latter assumes gradually, especially on warming, a bright raspberry-red colour. Under identical conditions pure dimethyl sulphate fails to react with either substance.

When the colouring matters were examined in chloroform solution, they were found to possess characteristic spectra.

An interesting reaction occurs in the presence of oxidising agents, of which ferric chloride is the most convenient for practical purposes. On addition of a drop of ferric chloride in glacial acetic acid solution, the bright raspberry-red colour of the cholesterol reaction changes into purple, while the purple of the "oxycholesterol" reaction changes into a brilliant emerald green. The latter shows a well-defined and characteristic absorption band in the red between λ 650–λ 630, which is identical with that obtained in the Lifschütz reaction [1908]. I found further that *pure* dimethyl sulphate in the presence of ferric chloride also gave the same emerald green colour with "oxycholesterol," while cholesterol under the same conditions gave no colour reaction even on prolonged warming. These reactions can be used to detect "oxycholesterol" in presence of cholesterol, and also to estimate its quantity spectrometrically.

The striking difference between the behaviour of technical and of pure

[1] This reaction has since been successfully applied by Lamb [1914] in a study of the influence of atmospheric oxygen on the oxidation of cholesterol under histological conditions.

dimethyl sulphate was next investigated. The active substance in technical dimethyl sulphate which reacts with "oxycholesterol" and with cholesterol in the absence of ferric chloride is evidently an impurity and my experiments tend to show that it is monomethyl sulphate. This probably owes its origin to partial hydrolysis of dimethyl sulphate in a moist atmosphere, and its formation explains the fact that even inactive *pure* dimethyl sulphate gradually becomes active on keeping under ordinary laboratory conditions.

The dimethyl sulphate reaction cannot serve for the distinction of animal and vegetable cholesterols, as I have found that sitosterol as well as "oxysitosterol" give colour reactions identical with those of cholesterol and "oxycholesterol" respectively.

Experimental.

The cholesterol used was prepared from human brain by the method of O. Rosenheim [1906]. "Oxycholesterol" was prepared from it by treatment with benzoyl peroxide in glacial acetic acid solution, according to Lifschütz [1913]. The spectroscopic observations were made with a Zeiss comparison spectroscope. The depths of the fluids examined were measured in a special absorption vessel, which allows measurements to 0·05 mm. A small electric arc lamp, at 20 cm. distance from the apparatus and screened by a plate of matted glass, served throughout the experiments as the source of light. The width of the spectroscope slit was 0·2 mm. in all observations.

I. *Reaction with technical dimethyl sulphate.*

(*a*) *Cholesterol.* On adding a few drops of technical dimethyl sulphate (Kahlbaum) to a solution of cholesterol in chloroform, the solution remains colourless for some minutes, but on slightly warming in a water-bath it gradually assumes a bright raspberry-red colour[1]. In sunlight, or in the light of the electric arc, it shows a blue fluorescence. Solutions of cholesterol in carbon tetrachloride, benzene, toluene or xylene give similar colour reactions, the colours tending to be more blue, less transparent and more fluorescent. Solutions in ether, alcohol, acetone, amyl acetate and amyl alcohol failed to give any reactions. The colour of the chloroform solution is discharged by the addition of water or alcohol. This fact distinguishes the reaction from that of Neuberg and Rauchwerger [1904] with methylfurfural, which is carried out in alcoholic solution. This also gives a raspberry-red solution, but shows a different spectrum.

[1] This reaction was discovered accidentally by Dr O. Rosenheim when carrying out experiments on the methylation of cholesterol.

On adding a drop of ferric chloride solution (5 % ferric chloride in glacial acetic acid), the colour is intensified and changes into a deep purple. The reaction is distinguished by great permanency and was observed to persist for more than a week.

For the spectroscopic examination 0·2 cc. of Kahlbaum's dimethyl sulphate (techn.) was added to 2 cc. of a 5 % cholesterol solution in dry chloroform. A deep raspberry-red colour developed on slight warming which showed two characteristic bands, one between *D* and *E* and the other near *F*. The following observations in the absorption vessel were made:

Depth of fluid

5·0 mm. only red light transmitted.
3·0 „ one dense band in yellow and green from λ 595–λ 460.
1·5 „ one dense band in yellow and green from λ 585–λ 485.
1·0 „ two bands from λ 575–λ 525 and from λ 505–λ 488.
0·7 „ „ from λ 565–λ 535 and from λ 505–λ 485.
0·5 „ „ from λ 560–λ 550 and from λ 500–λ 490.
0·4 „ one band at λ 500.

The appearance of the two characteristic bands is shown in Fig. 1 (Plate III).

When one drop of the ferric chloride reagent was added, the solution became so intensely coloured that accurate observations were impossible without dilution. The experiment was therefore repeated using a 1 % solution of cholesterol. On addition of ferric chloride the solution became purple and transmitted only red light when examined in 5 mm. thickness.

The following observations were made:

Depth of fluid

5·0 mm. only red light transmitted.
1·3 „ blue light also is transmitted.
1·0 „ two bands from λ 540–λ 530 and from λ 510–λ 420.
0·5 „ one band only from λ 505–λ 480.

The reaction is of approximately the same sensitiveness as Liebermann's cholesterol reaction, the limit being reached when 2 cc. of the solution contained 0·02 mg. of cholesterol.

(*b*) "*Oxycholesterol.*" A solution of this substance in chloroform gives on the addition of a few drops of technical dimethyl sulphate an immediate purple colour without warming (stage 1). A drop of the ferric chloride reagent added changes this at once into a greenish blue which gradually turns into an emerald green (stage 2) showing a green fluorescence. The

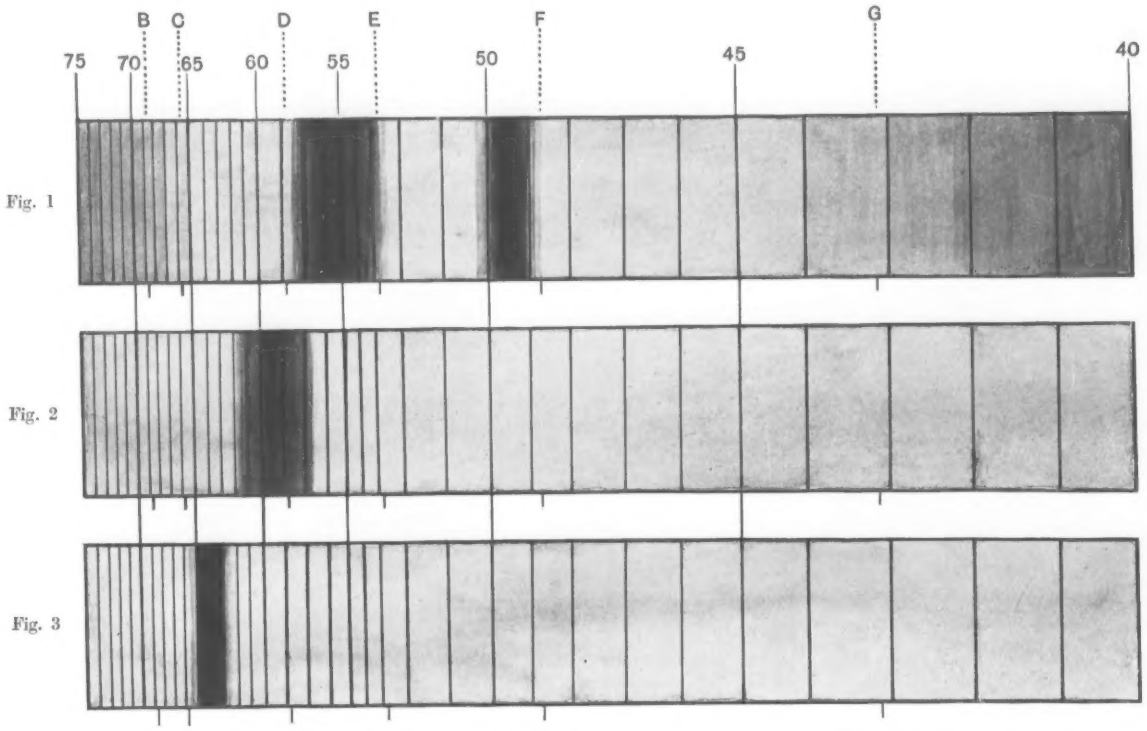


Fig. 1. Spectrum of Cholesterol Reaction, 0·02 g. in 2 cc. chloroform, thickness of layer: 1 mm.
Fig. 2. Spectrum of "Oxycholesterol" Reaction, 1st stage 0·02 g. in 2 cc. chloroform, thickness of layer: 0·5 mm.
Fig. 3. Spectrum of "Oxycholesterol" Reaction, 2nd stage, 0·02 g. in 2 cc. chloroform, thickness of layer: 0·5 mm.

green colour is discharged by water, acetone, alcohol or amyl alcohol, but not by glacial acetic acid, chloroform or benzene. Glacial acetic acid appears to be the most suitable diluent, the solution remaining transparent, whilst chloroform or benzene gives rise to some turbidity.

For spectroscopic examination 0·2 cc. of dimethyl sulphate (techn.) was added to 2 cc. of a 1 % "oxycholesterol" solution. Observations could only be made in thicknesses from 1·8 mm. downwards. All the spectral colours pass through the solution and there is only one band near the *D* line which shows best in a thickness of from 0·5 to 0·3 mm.

Depth of fluid

1·8 mm. one deep broad band from λ 620 to λ 520.
0·5 „ one broad band; blue, green and red light transmitted brilliantly.
0·3 „ one distinct band in the yellow, from λ 600—λ 580.
0·15 „ one faint band at *D* line—at λ 590.

The characteristic band of the first stage is shown in Fig. 2 (Plate III). On addition of two drops of glacial acetic acid and one drop of the ferric chloride reagent, the solution becomes greenish blue at once and rapidly reaches the second stage of emerald green. This also shows one band which is however in the red. The solution had to be diluted with an equal volume of acetic acid before satisfactory measurements could be made. The following observations were made with a freshly prepared solution containing only 0·5 % of "oxycholesterol."

Depth of fluid

10·0 mm. absorption of red end of spectrum as far as λ 560.
6·5 „ red light transmitted; blue and green come through undiminished.
5·0 „ one band from λ 670–λ 630.
4·0 „ one sharply defined band from λ 660–λ 630.
3·0 „ „ „ „ „
1·2 „ one band still shows faintly at λ 650.

Fig. 3 (Plate III) shows the typical band of the second stage in a 1·0 % solution of 0·5 mm. thickness.

The sensitiveness of the "oxycholesterol" reaction is somewhat less than that of the cholesterol reaction under the same conditions. The limit is reached when 0·2 mg. of "oxycholesterol" is contained in 2 cc. of chloroform; but on the other hand, the colour once produced in a stronger solution stands an amount of dilution which is much greater than the above limit.

The formation of "oxycholesterol" from cholesterol may be very well shown by means of the above reaction, when a small quantity of cholesterol is boiled in acetic acid solution with benzoyl peroxide, according to Lifschütz [1908]. On the addition of a few drops of dimethyl sulphate (techn.) a brilliant blue colour develops at once, which changes into green on standing, or immediately after the addition of a drop of ferric chloride solution. The green solution shows the single band in the red of the "oxycholesterol" reaction in the typical manner.

II. *Reaction with pure dimethyl sulphate.*

(*a*) *Cholesterol.* Pure dimethyl sulphate does not give rise to any colour reaction with either dry cholesterol or its solutions in chloroform or other solvents. The solutions remain colourless also on warming in a water-bath or after the addition of a drop of the ferric chloride reagent.

(*b*) "*Oxycholesterol.*" Strong "oxycholesterol" solutions in chloroform show, after the addition of pure dimethyl sulphate and warming in the water-bath, a slight purple colour which changes after the addition of ferric chloride into the typical green colour, showing the characteristic single band in the red. Even dilute "oxycholesterol" solutions which remain colourless with pure dimethyl sulphate, still turn green after the addition of ferric chloride, and show the "oxycholesterol" band in the red.

III. *The nature of the active substance in technical dimethyl sulphate.*

The fact that pure dimethyl sulphate fails to react with cholesterol makes it evident that the active substance in the technical product is of the nature of an impurity. An attempt was made to isolate this impurity by fractional distillation, but it was found that all the fractions as well as the residue in the distilling flask gave equally strong reactions. It was next observed that inactive pure dimethyl sulphate became active when it was simply heated to boiling in a test tube. At the same time it became strongly acid to Congo paper, while it was previously neutral. It was further noticed that a sample of inactive pure dimethyl sulphate, which had been kept for a considerable time in a bottle under ordinary laboratory conditions, had become active and had at the same time acquired an acid reaction. A control sample, kept in a sealed tube for the same length of time, had remained inactive. Further, it was found that all the samples of active technical dimethyl sulphate examined possessed an acid reaction to Congo paper.

It is well known that dimethyl sulphate is partially decomposed into methyl alcohol and monomethyl sulphate on heating to the boiling point, as well as by partial hydrolysis, and monomethyl sulphate may be further hydrolysed into methyl alcohol and sulphuric acid. The latter cannot be considered as the active substance since the addition of small quantities of concentrated sulphuric acid to pure dimethyl sulphate failed to activate it. Further, sulphuric acid gives rise to the well-known Salkowski's reaction, from which the dimethyl sulphate reaction is characteristically different, both in its appearance and in its behaviour towards ferric chloride.

It seemed therefore most likely that the acid monomethyl sulphate was responsible for the reaction. Since this substance forms a barium salt which is insoluble in chloroform, an indirect proof for this assumption may be obtained. A sample of technical dimethyl sulphate, dissolved in chloroform, was treated with solid barium carbonate until the effervescence had ceased. The filtered solution was found to be inactive and left, on careful evaporation of the chloroform, an oil which reacted neutral towards Congo paper. The inactive dimethyl sulphate thus prepared was easily activated again by boiling it for a short time in a test tube. Since the preparation of pure monomethyl sulphate from its barium salt is a matter of some difficulty, I have refrained from further testing this question.

There remained still the possibility that the activity of technical dimethyl sulphate might be due to traces of formaldehyde or formic acid, formed by gradual oxidation of methyl alcohol which is set free by hydrolysis. Formaldehyde and formic acid were indeed found to give rise to colour reactions with cholesterol under certain conditions, which need not be described here. But pure dimethyl sulphate does not produce any colour reaction in the presence of a trace of formaldehyde or formic acid, and there is therefore no reason to ascribe to either of them any share in the reaction of technical dimethyl sulphate with cholesterol.

The reaction of cholesterol with monomethyl sulphate may be used conversely for testing the purity of dimethyl sulphate preparations. Since technical dimethyl sulphate is probably manufactured by distillation under ordinary pressure, most of the samples contain monomethyl sulphate in varying amounts, whilst a distillation *in vacuo* furnishes a pure product which does not react with cholesterol.

It may be mentioned that diethyl sulphate behaves in exactly the same way. The pure substance does not react with cholesterol, but with "oxycholesterol" gives rise to a typical green reaction with ferric chloride, showing

the characteristic absorption band between λ 650 and λ 630. By heating the pure product to boiling in a test tube it also became active towards cholesterol and then behaved just like technical dimethyl sulphate.

Cholesterol esters give the dimethyl sulphate reaction in a less degree but vegetable cholesterols (phytosterols) behave exactly like cholesterol. A phytosterol prepared from hemp-seed and sitosterol from wheat embryos (prepared according to Ritter [1902]) were tested. The corresponding "oxysitosterol" reaction also was spectroscopically indistinguishable from that of "oxycholesterol."

The relationship of cholesterol to the terpenes suggested an examination of the behaviour of some of these substances towards dimethyl sulphate. It was found that borneol, camphor and abietinic acid (prepared from colophony), as well as cholalic acid, all give rise to colour reactions with technical dimethyl sulphate, the description of which is outside the scope of this communication. But none of these substances, when subjected to oxidation with benzoyl peroxide, as in the preparation of "oxycholesterol," gave rise to the typical "oxycholesterol" reaction with dimethyl sulphate.

Summary.

1. A colour reaction of cholesterol with technical dimethyl sulphate is described, the spectrum showing two bands, from λ 560–λ 550 and from λ 500–λ 490.

2. This reaction is not given by *pure* dimethyl sulphate and appears to be due to the presence of monomethyl sulphate.

3. "Oxycholesterol" gives a purple colour reaction with technical dimethyl sulphate showing an absorption band in the yellow (λ 600–λ 580).

4. Pure or technical dimethyl sulphate, after the addition of ferric chloride, gives a typical green colour reaction with "oxycholesterol," showing a well-defined absorption band in the red between λ 650 and λ 630.

The expenses of this research have been defrayed by a grant from the Government Grant Committee of the Royal Society.

REFERENCES

Lamb, F. W. (1914). *J. Physiol.*, **48**; *Proceedings*, lvi.
Lifschütz, I. (1908). *Ber.*, **41**, 252.
—— (1913). *Biochem. Zeitsch.*, **48**, 373.
Neuberg, C. and Rauchwerger, D. (1904). *Festschrift f. E. Salkowski (Berlin)*, 279.
Ritter, E. (1902). *Zeitsch. physiol. Chem.*, **34**, 456.
Rosenheim, M. C. (1914). *Biochem. J.*, **8**, 74.
Rosenheim, O. (1906). *J. Physiol.*, **34**, 104.

XIX. THE DECOMPOSITION OF LACTIC ACID BY KILLED YEAST.

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(*Received March 27th, 1916.*)

According to the theory that the first stages both of anaerobic respiration and of alcoholic fermentation consist of a series of oxidations and reductions at the expense of water [Palladin, 1912], it must be concluded that amongst the first products of decomposition of glucose must be placed organic acids. These acids must undergo further anaerobic oxidation in which reductase participates. Palladin and Mlle Lovchinovskaya [1914] therefore investigated the action of a hydrogen acceptor (methylene blue) on the decomposition of some organic acids—supposed to be present among the intermediate products of the decomposition of glucose—by killed yeast. Of the acids investigated, gluconic and lactic caused the evolution of a greater quantity of carbon dioxide in the presence of methylene blue, than in its absence. The action of reductase consists in removing the hydrogen from one substance and transferring it to another. The substance which yields the hydrogen is called the oxidisable substance, or reductor ($R \,.\, H_2$), whereas the substance receiving the hydrogen is called the hydrogen acceptor, or oxidator (A):

$$R.H_2 + A = R + A.H_2.$$

If we take the case in which reduction takes place with the participation of water, the hydrogen of the water will join the hydrogen acceptor, and the oxygen will join the oxidisable substance and either remove from it hydrogen with the formation of water, or produce some other oxidising reaction.

The oxidations produced by reductase by means of removing the hydrogen may be accompanied by the evolution of carbon dioxide. Thus, Bredig and Sommer [1910] have shown that formic acid is decomposed into carbon dioxide and hydrogen in the presence of a catalyst and methylene blue:

$$HCO_2H + M = CO_2 + M.H_2.$$

The carbon dioxide produced in the decomposition of gluconic and lactic acids also owes its production to reductase. Having put off for some time the detailed investigation of gluconic acid, we turned to the study of the decomposition of lactic acid. The results of this research form the contents of the present paper.

We started from the supposition that in the presence of the hydrogen acceptor (A) lactic acid first produces pyruvic acid under the influence of reductase:

(1) $CH_3 . CH(OH) . COOH + A = CH_3 . CO . COOH + A . H_2$.

The pyruvic acid formed is then decomposed into carbon dioxide and acetaldehyde under the influence of carboxylase:

(2) $CH_3 . CO . COOH = CH_3 . CHO + CO_2$.

Beilstein and Wiegand [1885], thinking lactic acid to be a secondary alcohol and pyruvic acid to be the ketone corresponding to it,

$$\begin{matrix} CH_3 \\ COOH \end{matrix}\!\!>\!CH . OH \qquad\qquad \begin{matrix} CH_3 \\ COOH \end{matrix}\!\!>\!CO$$

obtained pyruvic acid by oxidising calcium lactate with potassium manganate. Wieland [1912] subsequently found that when palladium black acts on lactic acid, pyruvic acid is also produced. We worked with hefanol and zymin which were considerably weakened by rather long preservation. During the first half of our work we used methylene blue as a hydrogen acceptor, and in the second half acetaldehyde *in statu nascendi*.

I. The ratio of the quantity of acetaldehyde formed to the quantity of carbon dioxide evolved in presence of methylene blue.

In all the experiments of this work the lactic acid was neutralised by caustic potash. The reaction therefore follows the equations:

(1) $2CH_3 . CH(OH) . COOK + 2M = 2CH_3 . CO . COOK + 2MH_2$,

(2) $2CH_3 . CO . COOK + H_2O = 2CH_3 . CHO + CO_3K_2 + CO_2$.

As is seen, part of the carbon dioxide remains combined in the solution. In order to produce it in a free state, 10 drops of 3 % sulphuric acid were added after the termination of the experiment. In that case:

$$\frac{CO_2}{CH_3CHO} = \frac{44}{44} = 1.$$

The experiments were conducted in round-bottomed flasks with long necks, with a capacity of 50 cc. The flasks were closed with rubber corks with three holes. Through one hole a tube passed to the bottom, through

the second, a short tube. Both tubes carried at the top india-rubber tubes which were tightly closed during the experiment by means of stoppers. Into the third opening a funnel with a tap was fitted, through which the sulphuric acid was poured. During the experiment the flasks were shaken up in a special shaking apparatus. On this account even a partial decolorisation of the methylene blue was never observed. After the addition of sulphuric acid a current of air was conducted through the flask and then through a Pettenkofer's tube.

As qualitative reactions for acetaldehyde, the reaction with fuchsin-sulphurous acid and the reaction of Rimini were used. The quantitative determination of acetaldehyde was carried out by distillation into a flask cooled by snow. In the distillate the aldehyde was determined by adding bisulphite and titrating the excess by iodine according to Ripper's method.

Exp. 1. Three flasks. In each 7 g. of hefanol and 25 cc. of 0·5 % methylene blue. In the first and second 25 cc. of 2 % potassium lactate. In the third, 25 cc. of water. The duration of the experiment was 3 h. 20 m. Temperature 17–18°.

Results: No. 1. 75·0 mg. CO_2.
„ 2. 31·3 „ acetaldehyde.
„ 3. 40·6 „ CO_2.

After subtracting from 75·0 the carbon dioxide of auto-fermentation, we have 34·4. Therefore,

$$\frac{CO_2}{CH_3.CHO} = \frac{34{\cdot}4}{31{\cdot}3} = 1{\cdot}1.$$

Exp. 2. Repetition of the preceding. Duration 6 hrs. Temperature 17–18°.

Results: No. 1. 94·6 mg. CO_2.
„ 2. 46·2 „ acetaldehyde.
„ 3. 41·2 „ CO_2.

$94{\cdot}6 - 41{\cdot}2 = 53{\cdot}4,$

$$\frac{CO_2}{CH_3.CHO} = \frac{53{\cdot}4}{46{\cdot}2} = 1{\cdot}16.$$

Exp. 3. Repetition of preceding. Duration 7 hrs. Temperature 17–18°.

Results: No. 1. 99·1 mg. CO_2.
„ 2. 49·0 „ acetaldehyde.
„ 3. 48·2 „ CO_2.

$99{\cdot}1 - 48{\cdot}2 = 50{\cdot}9,$

$$\frac{CO_2}{CH_3.CHO} = \frac{50{\cdot}9}{49{\cdot}0} = 1{\cdot}04.$$

Thus, in all the three experiments the ratio CO_2/CH_3CHO is, according to the theory, nearly equal to one, if we hold that in the presence of lactic acid auto-fermentation continued with the same energy as in the control experiment.

The question arises, why was not the acetaldehyde further reduced to ethyl alcohol? It is quite possible that the presence of a large quantity of the hydrogen acceptor, which largely takes away the hydrogen, prevents this reaction. It may be expressed in the form of a general rule that *in the presence of a large quantity of the hydrogen acceptor the formation of alcohol is impossible.* If applied in the necessary manner, the hydrogen acceptor can be made to interrupt the alcoholic fermentation at the stage of acetaldehyde.

The experiments described above have already appeared in Russian in a paper by Palladin, Sabinin and Lovchinovskaya [1915] published on 1st May, 1915. In September there appeared a paper by Harden and Norris [1915], who, not knowing of our work, had also discovered that lactic acid is decomposed by dried yeast with the help of reductase and carboxylase, and that as a result acetaldehyde is produced. In this work there is mentioned the very interesting fact that only a small part of the oxidised lactic acid is converted into acetaldehyde. As Harden and Norris did not find any pyruvic acid, they suggested that possibly only part of the lactic acid was converted into pyruvic acid, the greater part oxidising otherwise. The following facts speak in favour of such a view: Palladin, Lovchinovskaya and Alexeieff [1915] discovered that methylene blue interrupts the decomposition of pyruvic acid by zymin and wheat germs. Further, according to the equation

$$2CH_3 . CO . COOK + H_2O = 2CH_3 . CHO + CO_2 + K_2CO_3,$$

it was to be expected that after the end of the experiment the quantity of carbon dioxide in the form of carbonate of potassium would be equal to the quantity of gaseous carbon dioxide evolved.

In reality it was different. In the control portion the combined carbon dioxide was less by 62 %, and in the experimental portion by 82 %, as is seen from the following experiment quoted from their work.

Exp. iv. Three portions of 5 g. of old zymin. 1. 50 cc. of 1 % pyruvic acid neutralised by caustic potash. Air current. 2. 50 cc. of 1 % pyruvic acid neutralised by caustic potash; 1·5 g. methylene blue. Hydrogen current. 3. 50 cc. water, 1·5 g. methylene blue. Hydrogen current. After 24 h. 10 m. the experiment was stopped. There was no decolorisation. Then 10 cc. 5 % H_2SO_4 were added to 1 and the same quantity to 2, 2 cc. of toluene in each flask. Temperature 14·5–15°.

	1 1 % potassium pyruvate. Air current		2 1 % potassium pyruvate. Methylene blue. Hydrogen current		3 Water. Methylene blue. Hydrogen current	
Time	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.
2 hrs.	32·2	16·1	22·2	11·1	4·6	2·3
2 „	25·8	12·9	14·2	7·1	4·2	2·1
20 „	41·1	2·0	38·7	1·9	7·0	0·3
24 „	99·1	—	75·1(−24%)	—	15·8	—
			H_2SO_4 added.			
3 hrs. 35 m.	38·6(−62%)	—	13·2(−82%)	—	—	—
Total	137·7	—	88·3	—	—	—

It is to be concluded from the evidence given in such experiments that parallel to the decomposition of both the pyruvic and the lactic acids, the formation of some acid or acids decomposing CO_3K_2 with the evolution of carbon dioxide takes place. In presence of methylene blue a greater quantity of these acids is formed, than in the control portions. Still less combined carbon dioxide is produced by decomposing lactic acid by killed yeast in the presence of methylene blue, as was shown in the paper by Palladin, Sabinin and Lovchinovskaya [1915] mentioned above. Lvoff [1913] discovered in our laboratory that in the process of glucose fermentation for each atom of hydrogen removed with the help of methylene blue, the production both of alcohol and of carbon dioxide is diminished by one molecule. This led Lovchinovskaya to try to find some similar laws for lactic acid.

According to the equations:

(1) $2CH_3 . CHOH . COOK + 2M = 2CH_3 . CO . COOK + 2MH_2$,

(2) $2CH_3 . CO . COOK + H_2O = 2CH_3COH + CO_3K_2 + CO_2$,

and taking into account that the molecular weight of methylene blue, $C_{16}H_{18}N_3SCl + 3H_2O$, is 373·5, it follows that the ratio

$$\frac{CO_2}{2M} = \frac{44}{747{\cdot}0}.$$

It follows from this, that for 1·5 g. of methylene blue before its complete decolorisation in an atmosphere of hydrogen there must be evolved 88·3 mg. of carbon dioxide. Experiments produced greater quantities.

Exp. 4. Three portions of 10 g. of hefanol. 1. 50 cc. 2 % lactic acid neutralised by caustic potash, and 1·5 g. methylene blue. Hydrogen current. 2. 50 cc. 2 % lactic acid neutralised by caustic potash. Air current. 3. 50 cc. of water. Air current. To each flask 2 cc. of toluene were added. After decolorisation of 1, the experiment was stopped. Temperature 16°.

Time	Potassium lactate and methylene blue. Hydrogen current		Potassium lactate. Air current		Water. Air current	
	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.
4 h. 45 m.	78·4	18·4	31·0	7·3	26·0	6·1
23 hrs.	177·6	7·7	63·6	2·7	53·0	2·3
24 h. 45 m.	256·0	—	94·6	—	79·0	—

256 − 94·6 = 161·4 instead of 88·3.

Similar experiments gave the following figures:

	161·4
	154·2
	139·6
	148·6
	108·8
	147·7
Average:	143·4

If we suppose that all the CO_2 had been evolved $88{\cdot}3 \times 2 = 176{\cdot}6$ mg. of CO_2 would have been obtained. In reality, the average was found to be 143·4, *i.e.* 81·2 %. Therefore, a small part of the carbon dioxide must have remained in a combined state, and this was confirmed by experiment.

Exp. 5. One portion of 11·5 g. dried yeast (Lebedeff); 50 cc. 1 % lactic acid neutralised by caustic potash; 1·5 g. methylene blue; 2 cc. of toluene. The experiment was stopped after 44 h. 20 m. There was no decolorisation. Afterwards 10 cc. 5 % H_2SO_4 were added. Temperature 14° C.

Duration of the experiment	Potassium lactate and methylene blue. Hydrogen current	
	CO_2 mg.	CO_2 in 1 h.
2 h. 30 m.	12·2	4·9
16 h. 10 m.	95·1	5·8
2 h. 40 m.	46·8	17·5
23 hrs.	58·0	2·5
44 h. 20 m.	212·1	—
Addition of H_2SO_4.		
24 hrs.	19·2	—
Total	231·3	—

Consequently, there appeared to be only 9 % of combined carbon dioxide.

II. The ratio of the quantity of carbon dioxide evolved to the quantity of oxygen absorbed in the presence of methylene blue.

The experiments in this part of our work were undertaken on the basis of the following equations:

(1) $2CH_3 . CHOH . COOK + 2M = 2CH_3 . CO . COOK + 2MH_2$,

(2) $2MH_2 + O_2 = 2M + 2H_2O$,

(3) $2CH_3 . CO . COOK + H_2O = 2CH_3 . CHO + CO_3K_2 + CO_2$,

from which it follows that

$$\frac{CO_2}{O_2} = 1.$$

But the experiments of Lovchinovskaya proved that not merely half of the carbon dioxide is evolved, according to the equation, but a considerably greater amount. Therefore, the ratio CO_2/O_2 ought to be much larger than one. Nevertheless, it proved to be equal to one. This shows that, besides oxidising the reduced methylene blue, the oxygen enters into some other oxidation. In the first experiments fermentation took place at the temperature of the room in flat conical flasks similar to those used in cultivating diphtheria. The flasks were sealed by mercury, with two tubes—sealed by mercury—for removing the gas. During the experiment the flasks were shaken once or twice. This proved to be insufficient, the methylene blue having become partly decolorised at the end of the experiment, and high coefficients having been produced.

Exp. 6. Two flasks. In each 2 g. of hefanol and 30 cc. 1 % potassium lactate. In addition, in the second 3 cc. 0·5 % methylene blue, and in the first 30 cc. water. Duration of the experiment 5·5 hours. In all the experiments the gas was analysed in the Polovtzeff-Richter apparatus.

Flask No. 1	Flask No. 2
CO_2 = 3·5 %	CO_2 = 1·41 %
O_2 = 19·3 %	O_2 = 19·92 %
N_2 = 77·2 %	N_2 = 78·67 %
CO_2/O_2 = 4·5 %	CO_2/O_2 = 1·65 %

Exp. 7. Repetition of the preceding. Duration 5 hrs. 25 cc. of each liquid.

Flask No. 1	Flask No. 2
CO_2 = 2·8 %	CO_2 = 1·56 %
O_2 = 19·29 %	O_2 = 20·04 %
N_2 = 77·91 %	N_2 = 78·4 %
CO_2/O_2 = 2·86 %	CO_2/O_2 = 2·21 %

Exp. 8. Two flasks. In each 5 g. of hefanol and 25 cc. 2 % potassium lactate. In addition, in the first 25 cc. 0·5 % methylene blue, and in the second 25 cc. water. In the thermostat at 34°. Duration of the experiment 3·5 hrs.

Flask No. 1	Flask No. 2
CO_2 = 7·83 %	CO_2 = 3·84 %
O_2 = 18·01 %	O_2 = 19·53 %
N_2 = 74·16 %	N_2 = 76·63 %
CO_2/O_2 = 4·90 %	CO_2/O_2 = 5·48 %

Notwithstanding the very thin layer of liquid, in the last three experiments the aeration was very incomplete, and high coefficients were produced. In the experiments succeeding the flasks used in the experiments 1–3 were taken. During the experiment they were shaken on a shaking apparatus.

Exp. 9. Three flasks. Duration of experiment 3 hrs.

	Flask No. 1	Flask No. 2	Flask No. 3
Hefanol	5 g.	5 g.	5 g.
Methylene blue 0·5 % ...	25 cc.	25 cc.	—
Potassium lactate 2 % ...	25 cc.	—	25 cc.
Water	—	25 cc.	25 cc.

Analysis of gases:

Flask No. 1	Flask No. 2	Flask No. 3
CO_2 = 7·74 %	CO_2 = 3·72 %	CO_2 = 3·84 %
O_2 = 15·31 %	O_2 = 19·50 %	O_2 = 19·04 %
N_2 = 76·95 %	N_2 = 76·78 %	N_2 = 77·12 %
CO_2/O_2 = 1·54 %	CO_2/O_2 = 4·83 %	CO_2/O_2 = 2·90 %

Allowing for the control experiment (No. 2) we have as the final ratio

$$\frac{7{\cdot}74 - 3{\cdot}72}{5{\cdot}00 - 0{\cdot}77} = 0{\cdot}95.$$

Exp. 10. Three flasks. Duration of experiment 3·5 hrs.

	Flask No. 1	Flask No. 2	Flask No. 3
Hefanol	7 g.	7 g.	7 g.
Methylene blue 0·5 % ...	25 cc.	25 cc.	—
Potassium lactate 2 % ...	25 cc.	—	25 cc.
Water	—	25 cc.	25 cc.

Analysis of gases:

Flask No. 1	Flask No. 2	Flask No. 3
CO_2 = 9·84 %	CO_2 = 4·63 %	CO_2 = 5·77 %
O_2 = 13·45 %	O_2 = 18·44 %	O_2 = 18·91 %
N_2 = 76·71 %	N_2 = 76·93 %	N_2 = 75·32 %
CO_2/O_2 = 1·44 %	CO_2/O_2 = 2·47 %	CO_2/O_2 = 5·94 %

Allowing for the control as above we have:

$$\frac{CO_2}{O_2} = 1{\cdot}06.$$

Exp. 11. Three flasks. Duration of the experiment 5 hrs.

	Flask No. 1	Flask No. 2	Flask No. 3
Hefanol	7 g.	7 g.	7 g.
Methylene blue 0·5 % ...	25 cc.	25 cc.	—
Potassium lactate 2 % ...	25 cc.	—	25 cc.
Water	—	25 cc.	25 cc.

Analysis of gases:

Flask No. 1	Flask No. 2	Flask No. 3
$CO_2 = 12{\cdot}96\ \%$	$CO_2 = 4{\cdot}92\ \%$	$CO_2 = 6{\cdot}09\ \%$
$O_2 = 11{\cdot}24\ \%$	$O_2 = 18{\cdot}07\ \%$	$O_2 = 18{\cdot}40\ \%$
$N_2 = 75{\cdot}80\ \%$	$N_2 = 77{\cdot}01\ \%$	$N_2 = 75{\cdot}51\ \%$
$CO_2/O_2 = 1{\cdot}48\ \%$	$CO_2/O_2 = 2{\cdot}18\ \%$	$CO_2/O_2 = 3{\cdot}98\ \%$

Allowing for the control, we have:

$$\frac{CO_2}{O_2} = 0{\cdot}80.$$

The results of the last three experiments show that with good aeration the ratio CO_2/O_2 during the decomposition of potassium lactate by yeast in the presence of methylene blue is only slightly less than one.

Just as in respiration, according to the theory of Palladin, the oxygen is absorbed in order to remove the hydrogen; in the last three experiments the oxygen absorbed oxidised the hydrogen which was removed from the lactic acid by means of methylene blue. Thus, the methylene blue has taken the place of the respiratory pigment.

III. The decomposition of lactic acid in the presence of pyruvic acid.

The problem for our further experiments was to find the conditions in which the lactic acid would produce, instead of acetaldehyde, alcohol. The question then arose, what was the substance that in natural conditions substituted methylene blue during alcoholic fermentation in the process of decomposition of lactic acid? Of course, only acetaldehyde can be this substance. From our point of view the scheme of decomposition of lactic acid by yeast with the production of alcohol is represented in the following manner:

(1) $CH_3 . CH(OH) . COOH + CH_3 . CHO = CH_3 . CO . COOH + CH_3 . CH_2 . OH$,

(2) $CH_3 . CO . COOH = CO_2 + CH_3 . CHO$.

The acetaldehyde produced oxidises new quantities of lactic acid, and so on. Consequently, in the presence of an insignificant quantity of acetaldehyde it is possible to convert an indefinitely large quantity of lactic acid into

alcohol. In order to test the correctness of the view expressed, we took, instead of acetaldehyde, pyruvic acid, which when decomposed by carboxylase, produces acetaldehyde:

(1) $CH_3 . CH(OH) . COOH + CH_3 . CO . COOH = CH_3 . CH(OH) . COOH + CH_3 . CHO + CO_2$,

(2) $CH_3 . CH(OH) . COOH + CH_3 . CHO + CO_2 = CH_3 . CO . COOH + CH_3 . CH_2 . OH + CO_2$.

We had two reasons for taking pyruvic acid. Firstly, because it produces acetaldehyde *in statu nascendi*; secondly because Oppenheimer [1914] demonstrated that pyruvic acid evokes the decomposition of lactic acid by yeast juice.

Exp. 12. Nine flasks with 5 g. of zymin in each. In addition, in three flasks 25 cc. 0·2 % pyruvic acid neutralised by caustic potash, and 25 cc. of water; in three flasks 25 cc. 2 % lactic acid neutralised by caustic potash, and 25 cc. of water; in the remaining three flasks 25 cc. 2 % lactic acid, and 25 cc. 0·2 % pyruvic acid neutralised by caustic potash. The following quantities of carbon dioxide in mg. were evolved:

	18 hours	24 hours	60 hours
1. Pyruvic acid	116	174	248
2. Lactic acid	117	164	219
3. Lactic and pyruvic acids	147	223	325

The average quantities of carbon dioxide evolved in one hour were as follows:

Duration of the experiment. Hours	Pyruvic acid		Lactic acid		Lactic and pyruvic acids	
	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.	CO_2 mg.	CO_2 in 1 h.
18	116	6·5	117	6·5	147	8·2
6	58	9·5	47	7·8	76	12·6
36	74	2·0	55	1·5	102	2·8
60	248	—	219	—	325	—

Thus, the addition of a small quantity of pyruvic acid increased the evolution of carbon dioxide at the expense of lactic acid in the following manner:

18 hours. 147 − 117 = 30 (+ 25·6 %)
24 hours. 223 − 164 = 59 (+ 36·0 %)
60 hours. 325 − 219 = 106 (+ 48·4 %)

Taking into account, that from the decomposition of the pyruvic acid only 25 mg. of carbon dioxide could be evolved, it follows that the remaining 81 mg. (106 − 25 = 81) of carbon dioxide were evolved at the expense of the lactic acid.

Therefore, it is to be concluded that *pyruvic acid evokes the decomposition of lactic acid by killed yeast, though in a weaker degree than methylene blue.*

This surplus of carbon dioxide cannot be attributed to the stimulation of auto-fermentation by the pyruvic acid, as, in comparison with the quantity of carbon dioxide evolved in presence of lactic acid, in the experiments in which auto-fermentation proceeded in the presence of pyruvic acid only a slight surplus was produced, hardly exceeding the 25 mg. produced from the decomposition of pyruvic acid:

18 hours.	116 – 117 = 0
24 hours.	174 – 164 = 10
60 hours.	248 – 219 = 29

It must also be noted that the pyruvic acid stimulated the decomposition of lactic acid at first in a weak degree (25·6 %), but at the end of the experiment this stimulation gradually increased (48·4 %).

Exp. 13. Three flasks with 5 g. zymin in each. In addition, in the first flask 25 cc. 0·2 % pyruvic acid neutralised by caustic potash and 25 cc. of water, in the second flask 25 cc. 2 % lactic acid neutralised by caustic potash and 25 cc. of water, in the third flask 25 cc. of 2 % lactic acid and 25 cc. 0·2 % pyruvic acid neutralised by caustic potash. During fermentation the flasks were firmly closed with india-rubber stoppers. After 25 hours the quantity of acetaldehyde was estimated:

1.	Pyruvic acid...	13·5 mg.
2.	Lactic acid	0 „
3.	Lactic and pyruvic acids ...	11·5 „

In the third portion, according to the quantity of carbon dioxide evolved in the first experiment, it was to be expected that 59 mg. of acetaldehyde would be produced. But in reality only 11·5 mg. were produced.

It is to be concluded, therefore, that *the decomposition of lactic acid by killed yeast in the presence of pyruvic acid takes place without acetaldehyde being produced, i.e. in a different manner from that which takes place in the presence of methylene blue.*

Exp. 14. Two flasks with 5 g. zymin in each. In addition, in the first flask 25 cc. 2 % lactic acid neutralised by caustic potash and 25 cc. of water; in the second flask 25 cc. 2 % lactic acid and 25 cc. 0·2 % pyruvic acid neutralised by caustic potash. The experiment was conducted for 24 hours. In order to liberate the combined carbon dioxide, at the end of the experiment 10 cc. 10 % sulphuric acid were added to each of the two flasks. The quantity of alcohol was then estimated by the method of Nicloux.

	Carbon dioxide	Alcohol
1. Lactic and pyruvic acids	205	167
2. Lactic acid	138	143
	67	24

$$\frac{C_2H_6O}{CO_2}=\frac{24}{67}=0{\cdot}35.$$

Exp. 15. Three flasks with 7 g. zymin in each. In addition, in the first flask 25 cc. 0·2 % pyruvic acid neutralised by caustic potash and 25 cc. of water, in the second flask 25 cc. 2 % lactic acid neutralised by caustic potash and 25 cc. of water, in the third flask 25 cc. 2 % lactic acid and 25 cc. 0·2 % pyruvic acid neutralised by caustic potash. The experiment was conducted for 62 hours. The estimations of carbon dioxide and alcohol were made as in the preceding experiment.

	Carbon dioxide	Alcohol
1. Pyruvic acid	490	476
2. Lactic acid	447	445
3. Lactic and pyruvic acids	695	514

Therefore, the third portion gave the following surplus of carbon dioxide and alcohol, as compared with the second:

Carbon dioxide 695 – 447 = 248 mg.
Alcohol ... 514 – 445 = 69 „

If we suppose that in the third portion the pyruvic acid not only evoked the decomposition of lactic acid, but also stimulated auto-fermentation, we must subtract from the figures of the third portion those of the first portion, not of the second:

Carbon dioxide 695 – 490 = 205 mg.
Alcohol ... 514 – 476 = 38 „

Therefore, even if we allow for the stimulation of the process of auto-fermentation by pyruvic acid in the presence of lactic acid, we must conclude that *pyruvic acid evokes the decomposition of lactic acid by killed yeast with the production of alcohol and carbon dioxide.* But a sharp deviation from the normal alcoholic fermentation is observed, for to a great quantity of carbon dioxide evolved, only a small quantity of alcohol is produced:

$$(1)\quad \frac{C_2H_6O}{CO_2}=\frac{69}{248}=0{\cdot}27,$$

$$(2)\quad \frac{C_2H_6O}{CO_2}=\frac{38}{205}=0{\cdot}18.$$

What has caused such a great predominance of carbon dioxide over alcohol? It is well known from organic chemistry that reactions very rarely correspond to the theoretical equations: usually the main reaction is accompanied by a greater or smaller number of accessory reactions. Moreover, all the

chemical reactions studied by us in killed organisms differ more or less widely from the same reactions which take place in living organisms, in that they are accompanied by different accessory reactions, absent from living organisms.

In all probability, in the case investigated by us, the accessory reactions have also partly obscured, and partly altered, the main reaction of decomposition of lactic acid into alcohol and carbon dioxide. It is possible, that one of these accessory reactions was the reaction of Cannizzaro, very common in killed plants and animals. It is unknown whether it takes place in living organisms. This reaction alone could have reduced the production of alcohol by half. It may be also that part of the lactic acid was decomposed with the evolution of carbon dioxide, but without the formation of the aldehyde. Lastly, it is possible that the surplus of carbon dioxide was produced by further oxidation of the acetaldehyde. Further investigations are needed to solve these questions.

A surplus of carbon dioxide over alcohol is a usual phenomenon during anaerobic respiration of seminiferous plants. It is to be hoped that some of these cases will be explained after further studies on the decomposition of lactic acid by yeast in the presence of pyruvic acid.

The alcohol which was found in portions with lactic acid alone, was, of course, not produced from the latter, but appeared as the result of auto-fermentation, as the control experiment proved that the addition of neutralised lactic acid to zymin scarcely increases the quantity of carbon dioxide evolved and alcohol produced. Thus, 7 g. of zymin after 62 hours of auto-fermentation evolved 400 mg. of carbon dioxide and produced 417 mg. of alcohol.

Just as the negative results of Buchner's experiments do not prove that lactic acid cannot be the intermediate product of alcoholic fermentation; our own positive results—on account of the discovery of the wide capacity of killed yeast for decomposing the most dissimilar compounds, which are known to have no relation whatever to alcoholic fermentation[1]—do not yet conclusively prove that lactic acid is the intermediate product of alcoholic fermentation. It remains to stop the alcoholic fermentation in the stage of lactic acid, and this is what we are now engaged upon. At any rate, our experiments give, firstly, a scheme which shows in which direction the work must be conducted, and, secondly, prove that *alcohol does not present the result of decomposition, but the product of reduction of acetaldehyde by removing the hydrogen from one of the intermediate products of decomposition of glucose.*

[1] For example, it is impossible, on account of the formation of aniline from nitrobenzene by killed yeast, to say that these substances are the normal products of metabolism in yeast.

The investigations on the significance of hydrogen during alcoholic fermentation and respiration have made a complete revolution in our ideas on these processes. They have proved that the reactions of reduction are of main importance. Firstly, on them depend the anaerobic oxidations. Secondly they have made it clear that, *during the formation of the intermediate products, in the molecule of glucose the hydrogen is displaced, and not the oxygen*, as Hoppe-Seyler supposed [1876]. Thirdly, the chemical processes of alcoholic fermentation and respiration cannot be explained by reactions of decomposition alone, as the hydrogen is displaced not only within the confines of one molecule, but also passes from one molecule into another. This means that during alcoholic fermentation and respiration the molecules act on each other reciprocally. Grey's experiments [1913] on the anaerobic fermentation of glucose by normal *Bacillus coli communis* (Escherich) and a strain derived from the normal organism by selection with sodium chloroacetate also speak in favour of the theory that lactic acid presents an intermediate product in alcoholic fermentation. The normal bacteria produce besides lactic acid,—acetaldehyde, alcohol and carbon dioxide. But artificially selected bacteria decompose glucose only as far as lactic acid, without producing either acetaldehyde or carbon dioxide, and producing very little alcohol.

REFERENCES.

Beilstein and Wiegand (1885). *Berichte*, **17**, 890.
Bredig and Sommer (1910). *Zeitsch. physikal. Chem.*, **70**, 34.
Grey (1913). *Biochem. J.*, **7**, 359.
Harden and Norris (1915). *Biochem. J.*, **9**, 330.
Hoppe-Seyler (1876). *Archiv gesam. Physiol.* **12**, 1.
Lvoff (1913). *Zeitsch. Gärungsphysiol.*, **3**, 289.
Oppenheimer (1914). *Zeitsch. physiol. Chem.*, **93**, 235.
Palladin (1912). *Zeitsch. Gärungsphysiol.*, **1**, 91.
Palladin and Lovchinovskaya (1914). *Biochem. Zeitsch.*, **65**, 129.
Palladin, Lovchinovskaya and Alexeieff (1915). *Bull. Acad. Impér. Sci. Petrograd*, 589.
Palladin, Sabinin and Lovchinovskaya (1915). *Bull. Acad. Impér. Sci. Petrograd*, 701.
Wieland (1912). *Berichte*, **45**, 2606.

XX. NITROGEN DISTRIBUTION IN THE TISSUES AND SOME OF THE FACTORS WHICH INFLUENCE IT.

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(*Received April 17th, 1916.*)

Previous Work.

In the development as well as in the foundation of our knowledge the importance of technique can hardly be overestimated; a new method has been the sesame to many a hitherto closed door and has opened new fields for research. One need only refer to the discovery and elaboration of the methods for the determination and isolation of the individual amino acids by Fischer, Abderhalden and others, which threw a flood of light upon the structure of the protein molecule and revealed the astounding fact of its complete cleavage in the alimentary canal by the various processes of digestion. Linked to this was the necessary corollary that the animal organism was capable of resynthesising protein out of its cleavage products. There was everything in favour of the further assumption that it would be able to maintain itself and build up the required tissue protein if the protein of the food were entirely replaced by its cleavage products or by a mixture of its component amino acids. Abderhalden [1912, 1] in one of his papers, dealing with the synthetic capacities of the tissues, gives an account of the various steps by which he was able to feed a dog on completely cleaved protein and finally on an artificial mixture of amino acids for periods of eight and twelve days in two successive experiments; in both cases nitrogen equilibrium was established and there was a very slight increase in weight. An examination of these experiments shows that the trypsin digests from dog, ox and horse protein were more satisfactory substitutes for protein than the amino acid mixture, for the result of their administration was

frequently a marked retention of nitrogen accompanied by a gain in weight. A gliadin digest was markedly inferior to the animal digests doubtless owing to the relative amounts of the constituent amino acids differing widely from those of the tissues. One very interesting experiment [Abderhalden, 1913] demonstrated in a striking way that protein cleavage products were not only capable of replacing tissue waste but that they sufficed also for the new formation of tissue; a dog which had fasted for 26 days was shaved and then fed on a diet which contained all its nitrogen in the form of completely digested flesh. Not only did the dog put on 10 kilos. weight but it grew a new coat showing unmistakably that a synthesis of protein from the amino acid mixture had taken place. In some experiments which were carried out by Buglia [1912, 1] the meat in the dietary of young growing dogs was replaced by meat digests without affecting the retention of nitrogen or the gain in weight, provided that the carbohydrate in the food was given in a readily absorbable form. Frank and Schittenhelm [1910, 1911], in a series of experiments which will be referred to later, found that the digests of various forms of protein were almost as efficient as the proteins themselves, and in the case of one of the best utilised proteins, egg albumin, the digest was actually as efficient as the undigested albumin.

The attention of physiologists having been directed to the synthetic capacities of the organism, it was only a step further to inquire whether the amino acids themselves could be built up out of simpler forms of nitrogen. The work of Knoop and Kertess [1911] and of Embden and Schmitz [1912] acted as a fresh stimulus in this direction. Knoop and Kertess had found that the injection of an α-keto-acid or of an α-hydroxy-acid was followed by the appearance of the corresponding amino acid in the urine showing that nitrogen had been added on in the tissues. Embden and Schmitz perfused the isolated glycogen-free liver with the ammonium salt of an α-keto-acid and were able to recover from the perfusing fluid the corresponding amino acid, and they further obtained alanine after perfusing ammonium chloride through a glycogen-rich liver.

It is now generally accepted that glycocoll can be synthesised in the rabbit [Abderhalden and Hirsch, 1912, 1] but the results of replacing the protein of the food by an ammonium salt or urea have not been as satisfactory as might reasonably have been expected if the tissues can readily synthesise the amino acids necessary for the further construction of protein. The main workers in this field have been Grafe [1912, 1, 2, 1913, 1, 2, 3, 1914] and Abderhalden [1912, 1, 2, 1913]. Grafe seems

to have started out with the definite expectation of finding that some, at any rate, of the food protein could be replaced by nitrogen in the form of ammonia or urea, provided that an abundant carbohydrate fat diet was supplied, but the large number of experiments performed, coupled with the statement that some of them were not as clear as he wished, indicates that he had difficulty in convincing himself that an unequivocal synthesis had occurred. Grafe [Grafe and Schläpfer, 1912] planned his experiments with great care and the conditions under which they were carried out were practically always the same, viz., the animal was prepared by a previous fast of some days, following which there was a pre-period, in which the diet was nitrogen free and consisted of abundant carbohydrate and fat to the amount of 150 or more calories per kilo. body weight. The effect of this was of course to reduce the nitrogen metabolism to a minimum. The mean of the last three days of the pre-period and of the first three days of a similar after-period served as a measure of the endogenous metabolism, and was used to determine the nitrogen retention in the principal period during which nitrogen was added to the carbohydrate fat diet in the form of an ammonium salt or [Grafe and Turban, 1913] of urea. By this method Grafe was able to secure a well-marked reduction of a negative nitrogen balance and an occasional positive balance on particular days in the principal period. He claimed that very considerable amounts of nitrogen were retained, *e.g.* in one experiment [1912, 2] on a pig, 14 g. of ammonium citrate nitrogen were retained in 16 days. Young growing pigs proved more suitable for these experiments than dogs because they took the food readily and put on weight with astounding rapidity. It is noteworthy as showing how long life can be maintained in a state of complete nitrogen starvation, that two young pigs [1913, 3] of the same litter, one of which was fed on carbohydrate and fat alone, while urea and ammonium citrate were added to this diet in the case of the second, both died on the 67th day of the experiment although the animal which received no nitrogen began to fail much earlier than the other.

Grafe's [1913, 1] most favourable results were obtained in his later experiments by a modification of the procedure; after reducing the nitrogen metabolism to a minimum in the usual way, in a further pre-period he gave a small amount of protein equal to from 0·5 to 0·7 of the maintenance minimum, which was continued in the principal period during the administration of urea alone or of ammonium citrate and urea. The nitrogen balance remained negative in the pre-period in spite of the small quantity of protein

but it became definitely positive in the principal period, *e.g.* pig 8 had an average daily balance of −0·64 g. in the pre-period which became + 0·812 g. in the principal period. Grafe points out the importance of making the difference between the nitrogen fed in the form of ammonium salt and urea on the one hand and the protein on the other as great in amount as possible, stating that it should be 3·5 times the value of the maintenance minimum and he criticises the small amount (1·3 times the maintenance minimum) used by Abderhalden and Lampe [1913, 1]. It is interesting to note that Grafe [1913, 2] found a negative daily balance of − 0·18 g. in a pig fed for 40 days with 4·7 g. of urea nitrogen daily as the sole source of nitrogen. In a later paper [1913, 3] he ran parallel experiments with pigs of the same litter; pig 15 was given rather less than the maintenance minimum of protein during the principal period, pig 16, on the other hand, received in addition urea and ammonium citrate; both pigs gained in weight but the balance remained negative in pig 15 and became positive in pig 16. In a second experiment pig 17 got rather more than the maintenance minimum of protein in the principal period, while pig 18 received no protein but only ammonium citrate and urea; both pigs behaved in exactly the same way putting on weight and exhibiting a positive nitrogen balance.

Grafe [1914] found that a certain amount of nitrogen retention also occurred when an inorganic salt, ammonium chloride, was substituted for the organic salt, provided that it was given in small or medium doses; in larger doses it exerted an injurious influence. An endeavour to satisfy the nitrogen requirements of the animal by giving it in the form of nitrate [Grafe and Wintz, 1913] led to most contradictory results partly owing to the injurious effect of the nitrate itself. Abderhalden and Hirsch [1913] found that sodium nitrate was quantitatively excreted in the urine in the case of four dogs; in spite of this the nitrogen balance, exclusive of the nitrate, was affected favourably in two and unfavourably in the other two dogs.

Grafe [1913, 3] mentions three possibilities with regard to the nitrogen retained; ammonia may be retained as such, or the ammonia may be anchored, *i.e.* it enters into some form of combination not protein, and lastly ammonia may spare body protein in some way, *i.e.* ammonia is retained in some protein-like form. He decides in favour of the nitrogen being retained in a protein-like form.

Abderhalden and his co-workers Hirsch and Lampe carried out their experiments on the same general principles as Grafe, but the experiments

differed in various details and were not planned with an equal uniformity. It is often difficult and tedious to follow the tables owing to the frequent absence of the average daily balance calculated for the various periods under consideration. The first results were published in 1912, immediately after the appearance of the paper by Grafe and Schläpfer; they dealt with the effects of superimposing ammonium carbonate or ammonium citrate on an abundant carbohydrate fat diet. Although the nitrogen balance remained negative some of the added nitrogen was undoubtedly retained. Loss of weight continued in spite of the feeding with ammonia, but in two instances when a blood or flesh digest was added to the nitrogen-free diet the weight rose and the balance became definitely positive. Shortly after this there appeared an account [Abderhalden and Hirsch, 1912, 2] of two experiments which lasted 103 and 111 days respectively in which ammonium salts were added to a nitrogen-free diet. The difficulty of the continued administration of ammonium salts due to vomiting and diarrhoea necessitated the frequent interruption of the ammonia periods and the intercalation of fast days. Sometimes the condition of the animal was such that feeding with meat powder or flesh digest for some days had to be resorted to, a procedure which was invariably followed by immediate recovery. A retention of some of the ingested ammonia undoubtedly occurred, although the authors urged caution in the interpretation of the results on account of the variations which were present in the daily nitrogen balance. They drew attention to the fact that a temporary gain in weight might be exhibited under certain conditions by an animal fed on a nitrogen-free diet. With regard to the utilisation of urea [Abderhalden and Hirsch, 1912, 4] any retention of nitrogen in the dog was followed by a washing out. In an isolated experiment [Abderhalden and Lampe, 1913, 2] on a pig there was not even a temporary holding back of nitrogen for the negative daily balance was practically the same as that of the succeeding fast days; it may be mentioned however that the nitrogen metabolism had not been reduced to a minimum by a pre-period of a nitrogen-free diet. A pig [Abderhalden and Lampe, 1913, 1] fed for 28 days on ammonium acetate, on the other hand, showed a definite protein sparing action of the ammonia although the balance remained negative.

Abderhalden and Lampe [1912, 2] criticised Grafe's findings and methods and complained that they were unable to obtain such favourable results. In their own experiments they stated that a positive nitrogen balance or nitrogen equilibrium was only transitory and was followed by an increased output. They admitted that ammonium salts exercised a protein sparing

action but considered that the facts were best explained by the retention of the nitrogen in a non-protein form. They doubted [1912, 1] the existence of any marked synthetic capacity on the part of the tissues on the ground that experiments on dogs, in which the nitrogen was supplied in the form of gelatin and ammonia, failed to show a positive nitrogen balance although there was undoubted retention of nitrogen. They maintained that if the cells could synthesise amino acids a much better result should be gained if the task imposed on them were merely that of synthesising the few amino acids which were lacking in the gelatin, than if they were required to synthesise the whole series present in the protein molecule.

Following on this paper came another [Abderhalden and Hirsch, 1912, 3] in which the effect of gelatin plus ammonia was compared with that of erepton (flesh digest) on a three months old dog. A positive nitrogen balance and a gain in weight always occurred when erepton was fed, whereas the weight fell and the balance became negative when it was replaced by gelatin with ammonia. Four young dogs were then fed for periods of seven days on (1) gelatin and ammonium acetate, (2) erepton, (3) an artificial mixture of amino acids, (4) erepton and (5) ammonium acetate successively. The weight of the animals and their general condition only was noted; the weight rose in the two erepton periods, remained stationary during the feeding with amino acids and fell when ammonium acetate was given either with or without gelatin. The animals were cheerful and well during the erepton periods but they slept all day and seemed definitely unwell during the administration of ammonia.

It is quite apparent from a consideration of the foregoing experiments that even if the organism can synthesise amino acids from ammonia and the products of carbohydrate and fat metabolism, it does not readily do so, for flesh digests are undoubtedly more satisfactory than ammonium salts or urea as sources of nitrogen supply.

In connection with the utilisation of ammonium salts Underhill and Goldschmidt [1913, 1] carried out some useful work and pointed out the advisability of estimating the ammonia as well as the total nitrogen. The type of experiment differed from those just described, for the dogs were fed on an adequate mixed diet and the particular ammonium salt was administered on one day only in three separate portions in gelatin capsules. A radical difference was found in the behaviour of the organic and the inorganic salts; none of the organic salt was excreted as ammonia and it was apparently quantitatively converted into urea. About 20 to 25 per cent. of the

inorganic salt, on the other hand, was excreted as urea while the remainder appeared as ammonia in the urine. There was, moreover, an increase in the nitrogen output and a lag in the elimination of the inorganic salt for the ammonia in the urine was increased for 48 hours after its administration.

A similar experiment [1913, 2] carried out on a fasting dog which had been freed from glycogen gave results in complete accord with the above. They [1913, 3] finally tested the utilisation of ammonium salts with a non-nitrogenous diet using Grafe's method. Their results were in keeping with those of previous workers in the case of the organic salts, which caused a retention of nitrogen and did not increase the urinary nitrogen, but contrary to the conclusion of Grafe and Schläpfer they found that ammonium chloride was incapable of acting as a source of nitrogen supply for the nutritional needs of the body, and not only was there an increased excretion of nitrogen, but for some days following the administration of the salt the ammonia in the urine was increased.

The authors criticise Grafe's technique particularly with regard to the absence of catheterisation, but in view of the duration and the number of Grafe's experiments and the care and consistency which characterise them his main results cannot be called in question.

Taylor and Ringer [1913] pointed out that the presence of carbohydrates was not an obligatory factor in the retention of nitrogen from ammonia. The administration of a single dose of ammonium carbonate to a dog, which had previously fasted three days, was accompanied by a retention of a considerable portion of it, the result being confirmed by two repetitions, while the retention in the case of two phloridzinised dogs was even greater. They claim that two administrations of urea were followed by a quantitative elimination of the amide, but in view of the variation in the daily nitrogen balance such a result requires confirmation. They suggest that ammonia nitrogen is retained because of a reversed reaction that leads to its combination with the α-ketonic and α-hydroxy-acids to form amino acids, which may be used in the synthesis or sparing of body protein. It cannot be said that these experiments have disproved Grafe's contention that the administration of abundant carbohydrate is the surest way of demonstrating a retention of ammonia nitrogen in the normal dog and pig.

Henriques and Anderson [1914, 1] attacked the problem of the utilisation of ammonium salts and urea in another way which excluded the action of the intestinal wall and of the intestinal bacteria. Using their method of

permanent intravenous injection in the goat they reproduced Grafe's experiments, *i.e.* there was a daily injection of two litres of fluid containing glucose and salts in a pre- and after-period, and intercalated between the two was a period in which urea or an ammonium salt was added to the injection. Although there was a diminution of the negative balance, a lasting retention of nitrogen was absent and the evidence indicated a retention followed by a washing out. When a meat digest was added to the fluid a positive balance was at once obtained emphasising the superiority of digests over ammonium salts to which attention has already been drawn.

It must not be forgotten that a great deal of work on the utilisation of ammonium salts and amides has been carried out on the herbivora and in their case no doubt exists as to the effect on the nitrogen balance. Among the recent workers Kellner, Eisenkolbe, Flebbe and Neumann [1910] conclude from their experiments that asparagine and ammonium acetate can replace the protein necessary for maintenance but that they cannot be utilised for protein formation even in starving animals.

Morgan, Beger and Westhausser [1910] state that ammonium acetate can be utilised in no inconsiderable way for the maintenance of life and even for milk formation if it be added to a diet containing sufficient starch but very deficient in protein. In a later paper [1911] they found that the nitrogen contained in ammonium acetate and asparagine in a diet poor in protein but with sufficient nitrogen-free food was utilised to the extent of 32 per cent. not only for the maintenance of life but also for the production of milk, flesh and wool. Probably the intestinal bacteria play an important part in the utilisation of these simple nitrogenous bodies in the herbivora for the value of these as food stuffs is admitted.

The complexity of the problems of protein metabolism is indicated by the apparent difference which exists between the processes of tissue repair and growth; various workers have shown that food which suffices for repair is not necessarily capable of producing growth. McCollum [1911] reduced the metabolism of a pig to a minimum by feeding it on a scanty starch and salt diet after a preliminary fast. The creatinine nitrogen under these conditions constituted 1/5·5 of the total nitrogen and at this low level all the nitrogen was assumed to be of endogenous origin; the creatinine nitrogen $\times$ 5·5 therefore was used as a measure of endogenous metabolism in the subsequent calculations and it was possible to determine how much of this endogenous or tissue nitrogen had been replaced by the nitrogen in the substance fed. It was found that zein was utilised to the extent of 80 per

cent. on an average for repair, but even when given in excess there was no evidence of the formation of body tissue. The average utilisation of gelatin for repair was 50–60 per cent., whereas when caseinogen was the only protein fed there were increases of body protein to the extent of 20–25 per cent.

Osborne and Mendel [1912, 2] carried out experiments on the feeding of isolated proteins to rats. Gliadin, which contains relatively very little glycocoll, arginine, histidine or lysine and extremely large quantities of glutamic acid, proline and ammonia, was unable to support normal growth in young growing rats who were dependent on it for their sole nitrogen supply. The same diet however sufficed for the maintenance of the adult animals, which not only paired but also produced healthy young, which were suckled by the mother, so that in this case there must have been a synthesis in the maternal organism of the amino acids which were lacking in the gliadin. Of four young rats suckled by a gliadin-fed mother, the three which were taken away from her at the end of 30 days and were then fed on caseinogen, edestin and milk respectively, showed normal growth, while the fourth, which was left in the cage with the mother and had access only to the gliadin mixture, showed no growth. Hordein also was incapable of maintaining normal growth in contradistinction to caseinogen, edestin and glutenin. As Osborne and Mendel point out maintenance experiments alone are not sufficient to solve the problem of the biochemical value of dietaries. An account appeared elsewhere [1912, 1] of an elaborate investigation which led to an extension of the list of proteins which were capable of supporting normal growth. The soy bean was the only member of the Leguminosae containing a protein which fulfilled these conditions. Hordein and zein resembled gliadin in their inability to do so. With regard to the quantity of protein it was found that normal growth occurred if 18 per cent. of protein (or 14 to 15 per cent. calories) was present in the diet, but there was no increased growth if this quantity was increased. Frank and Schittenhelm [1910] investigated the question of the superiority of the "arteignes" protein of Michaud for tissue repair over allied forms of protein. They reduced the nitrogen metabolism of dogs to a very low level by the use of a nitrogen-free, carbohydrate-rich diet following a fast and then tested the relative values of dog, horse, pig, fish, goose and pigeon flesh. In two out of the five dogs, dog protein was the best utilised but in the other three dogs one of the other forms of protein held the first place. They concluded that "arteignes" protein had no definite superiority over other nearly allied proteins provided they could be readily broken down and that they yielded

much the same mixture of amino acids. Continuing this line of work [1911] they succeeded in maintaining without loss of weight two dogs for 6·5 and 4·5 months respectively on a diet in which the nitrogen minimum was given in the form of different types of animal protein or their digests. They found no marked difference in the utilisation of the different types of meat and the digests were almost as efficient as the undigested proteins. One dog with an intake of 1·2 g. protein was practically continuously in nitrogen equilibrium or else showed a positive balance, in the second dog a positive balance was not reached neatly so soon.

The method of introducing food stuffs intravenously has been practised by a number of workers and has had for its object the exclusion of the action of the intestinal wall. Buglia [1912, 2] injected meat digests intravenously in dogs and cited as evidence of their utilisation that only varying proportions were excreted in amino acid form in the urine. The experiments lack uniformity and are few in number but such a utilisation is in harmony with the results of the work of Folin and Denis [1912] and van Slyke and Meyer [1912, 1913, 1, 2, 3] who showed that the products of digestion were carried to the tissues as such without undergoing any preliminary change in the intestinal wall.

Henriques and Anderson [1913] elaborated the method of intravenous injection and were able to keep a goat alive for 20 days by the daily introduction of protein digests into the blood stream; the nitrogen balance under these conditions was frequently positive and a daily retention of 1 or 2 g. of nitrogen was quite common. Their use of this method in the investigation of the utilisation of urea and ammonia has already been referred to.

The same observers [1914, 2] used their method of permanent intravenous injection in goats after total extirpation of the bowel. They managed to keep such animals alive for $2\frac{1}{2}$ days. During this time the injection of trypsin + erepsin digests caused a considerable deposit of nitrogen, for example in goat 1 positive balances of 2·2 g. and 4·18 g. were obtained on the first and second day respectively.

Oehme [1914] injected meat digests intravenously on a single day in the case of two fed and two fasting dogs respectively, the diet of the former consisting of carbohydrate and fat. The amount of amino acid excreted unchanged in the urine was much smaller in the fed than in the fasting dog but even in the latter the protein cleavage products injected had been extensively utilised, for the maximum excretion of amino acid was only 29·5 per cent. of the amount injected. In view of the capacity of the tissues

to pick up amino acids, etc. from the blood stream the fact of a certain amount of the amino acid introduced not appearing within a given time in the urine certainly cannot be regarded as evidence of its utilisation. The real aim of the researches on the introduction of food stuffs intravenously was to obtain evidence that the intervention of the intestinal wall was not necessary and to disprove the hypothesis enunciated by Abderhalden of a synthesis of the products of digestion by the cells of the intestine.

When we come to the field of tissue analysis mention must first be made of the work of Grund [1910] and Diesselhorst [1911]. Grund endeavoured to find evidence of protein storage in the liver of the dog as it had been claimed by Seitz that such a storage of protein took place to a very marked extent in the liver of the hen. Grund estimated the total nitrogen, the total protein nitrogen and the total phosphorus in the liver, kidney and muscle of the fed and fasting animals, and compared the two values. When the total nitrogen was calculated as a percentage of the initial body weight, there was a great diminution in all these organs but particularly in the case of the liver as the following figures show:

	Liver	Kidney	Muscle
Mean of the fed dog ...	1·059 %	0·157 %	0·185 %
Mean of the fasting dog ...	0·570 %	0·098 %	0·129 %

If the percentage of total nitrogen in the liver of the fed and fasting animals be compared, however, the increase is in the fasting animal, due presumably to a diminution of glycogen in the latter which would lead to a relative increase in the total nitrogen content of the organ. Changes in the amount of moisture are not accountable for the increase. The kidney and muscle show practically no change in the percentage of total nitrogen. Grund mentions the fact that the ratio of "rest" nitrogen (= total soluble nitrogen) to total nitrogen expressed as a percentage of the latter showed great irregularities in the individual experiments but only moderate differences between the fasting and fed animals; the average figures are:

	Liver	Kidney	Muscle
Fed animal ...	11·6 %	15·7 %	15·1 %
Fasting animal ...	10·5 %	12·9 %	13·2 %

The ratio of the total phosphorus to the total nitrogen was practically constant in the organs of the fed and fasting animals. The difficulty of interpreting Grund's figures lies in the impossibility of getting a fixed basis for comparison, both the initial weight of the body and the weight of the individual organs

being subject to variation from various factors. As we shall see later it is much easier to detect changes in the "rest" nitrogen than in the total nitrogen.

Diesselhorst tried to determine whether the diet influenced the chemical composition of the tissues by a comparison of the muscles of a dog fed on a scanty carbohydrate fat diet and an abundant meat diet respectively. Two dogs were fed on the meagre nitrogen-free diet for $1\frac{1}{2}$ to 2 months, a hind leg was then amputated in the case of each animal and both were fed on an abundant meat diet for approximately three months. During this period they rapidly put on weight and at its termination they were killed. The main fact which emerges from the experiment is that the nitrogen : carbon ratio remains unaltered and is practically the same in the two dogs, 1 : 32 and 1 : 31·8; these figures agree closely with the ratio of 1 : 33·8 obtained by a previous worker, Stockhausen.

With the work of Folin and van Slyke we turn over a new page in the history of protein metabolism, for it has solved the problem of the form in which the products of digestion enter the blood stream and the theory of a resynthesis in the intestinal wall has been relegated to oblivion. Folin [Folin and Denis, 1912, 1] was the first to publish his results dealing with the application to the blood and tissues of his new micro-methods for the determination of total nitrogen, urea and ammonia nitrogen. In his investigation of the effects on the blood and tissues of the absorption of the products of protein digestion he selected the cat as being the most suitable animal for his experiments. After taking samples of blood and removing the gracilis muscle from one leg, he introduced into a ligatured loop of the intestine solutions of urea, glycocoll, egg albumin and a pancreatic digest respectively; samples of blood were withdrawn at varying intervals during the experiment and at its close the gracilis was removed from the other leg. The determination of the total non-protein nitrogen and the urea in the samples thus obtained showed that there had been a rapid absorption of the urea from the intestine, that it had entered into the blood stream unchanged and had actually accumulated in the muscle. In the case of the glycocoll, the increase of the non-protein nitrogen in the blood and muscle showed that it also had entered the blood stream and had travelled unchanged to the muscle, the urea content of the blood, however, was slightly increased towards the end of the experiment indicating a conversion of some of the glycocoll into this substance. In the same way there was evidence of the direct transference to the tissues of the egg albumin and protein digest. In a later paper [1912, 3] by allowing

a longer interval, 2·5 to 3 hours, to elapse before the final samples of blood and muscle were taken Folin gave ample demonstration of the conversion into urea of some of the absorbed alanine, glycocoll and peptone, for, in addition to the increase of the non-protein nitrogen, there was also an increase in the urea of the blood and tissues. The absorption of creatine and creatinine on the other hand was unaccompanied by any other change than the accumulation of the non-protein nitrogen in the blood and muscle. The author points out that the liver has not brought about any specialised deaminisation of the amino acids and emphasises the fact that the urea forming process is characteristic of all the tissues.

A few experiments [1912, 1] on the difference in the non-protein and urea nitrogen content of the blood in the fasting and the fed animals provided very scanty data for drawing satisfactory conclusions as to the influence of feeding. The amount of non-protein and urea nitrogen, however, in the blood of cats fed on a nitrogen-free, carbohydrate fat diet was undoubtedly less than in the case of meat-fed cats and this result was confirmed by data in a later paper [1912, 3] where very low values were obtained after feeding cats on a rice and cream diet.

Folin had always been interested in the question of the deaminisation of the products of protein digestion in the intestinal wall and he succeeded in demonstrating in a striking way that the ammonia in the portal blood did not originate in any such process. He [Folin and Denis, 1912, 2] compared the ammonia content of the blood in the mesenteric branches of the portal vein and discovered that it was two or three times greater in the blood coming from the large intestine than in that from the small intestine, owing to the extraordinarily high ammonia content of the faeces in the large intestine, particularly after a meat diet. This coupled with the fact that the injection of a pancreatic digest, glycocoll or asparagine caused no increase in the ammonia of the portal blood was conclusive proof of the source of the portal ammonia. In connection with the question of absorption Folin [Folin and Denis, 1912, 4, 5] also showed that a certain amount of absorption both from the stomach and large intestine occurred after the introduction of solutions of amino acids, urea or peptone.

Van Slyke's work dealing with the fate of protein digestion products in the body was made possible by the elaboration of a new method [1913, 1] for the determination of amino acid nitrogen and is of the highest importance as it gives a direct method of demonstrating the presence of amino acid nitrogen—in Folin's work the presence of the amino acid was only indirectly

inferred from the increase in the non-protein nitrogen. Van Slyke established the following facts in his first paper [van Slyke and Meyer, 1912], which was essentially preliminary. The blood of normal dogs, which have fasted for 20 to 24 hours, contains from 3 to 5 mg. amino acid nitrogen per 100 cc. When 12 g. of alanine were injected into the circulation of a 14 kilo. dog in 13 minutes, the added amount of amino acid nitrogen in the blood diminished very rapidly, only a relatively small portion of the alanine (1·5 g.) being excreted in the urine. It was calculated, on the assumption that the total volume of the blood was 1/20 of the body weight, that five minutes after the injection only 1·47 g. of alanine remained in the circulation. When alanine was injected into the small intestine its direct absorption was proved by the increase of the amino acid nitrogen in the blood of the mesenteric vein. The amino acid content of the blood five hours after a meal of 1 kilo. meat was practically doubled in amount. In view of these facts van Slyke is amply justified in his conclusion that the amino acids are not synthesised in the bowel wall but are offered to the tissues directly. The elaboration of these results in a later paper [van Slyke and Meyer, 1913, 1] showed that the amino acid nitrogen of the tissues was increased by an intravenous injection of amino acid. Apparently however there is a saturation point which sets the limit to absorption, as the authors say they were never able to force the figure for muscle up beyond 80 mg. per 100 g., the saturation point for liver is much higher and amounts to 125 to 150 mg. By increasing the length of the experiment [van Slyke and Meyer, 1913, 2] and comparing the amino acid content of samples of the blood and tissues taken 30 minutes after the cessation of the injection with that of samples taken several hours later, it was found that the amino acid content of the liver rapidly decreased. Although the latter might have been practically doubled in amount by the injection, the liver was able to desaturate itself completely in two or three hours, whereas the amino acid content of the muscle remained practically unaltered during this period. The disappearance of the amino acid from the blood was accompanied by an increase in the amount of urea.

A short study [van Slyke and Meyer, 1913, 3] of the effects of fasting and feeding on the amino acid content of the tissues lacks sufficient data to establish any very definite conclusions, in view of the variations present in both fasting and fed animals. The figures show that the amino acid nitrogen in the tissues of dogs fed on a protein-rich diet was not greater than the amount found in some of the fasting dogs. A nitrogen-free, abundant carbohydrate fat diet was not included in the list but it is extremely likely

in view of Folin's work that under such conditions the amino acid nitrogen of the blood and tissues would have been lower than in either the fasting or the fed animal.

The following researches owe their origin to the work of Folin or of van Slyke. In their attempts to prove that the liver is not the main site of urea formation and to belittle its activity in this direction, Fiske and Sumner [1914] performed some experiments on cats, in which they completely excluded the liver from the circulation and subsequently introduced an amino acid into the blood stream. Under these conditions they produced a certain amount of evidence that urea formation still goes on. They claim that the accumulation of urea per unit mass of blood and tissues is as great in the absence of shock when the liver and other abdominal viscera are excluded from the circulation as when they are in their normal relations. It may be pointed out that the initial variation in the urea content of the blood and tissues is astounding, and one is tempted to ask whether the small increase in the values in the case of the muscle is not within the limits of experimental error.

In conjunction with Karsner, Fiske had previously [1913] carried out a few perfusion experiments on the cat's liver, ammonium carbonate or glycocoll being added to the perfusing fluid, but the experiments are of little value. The failure to find evidence of urea formation from the glycocoll can be attributed to the short duration of the experiments and to the relatively small amounts of glycocoll used. The whole amount of ammonia perfused was not converted into urea, and simply from a consideration of the figures for the total soluble nitrogen the authors regard it as doubtful if the liver has bound any ammonia as such. It may be pointed out that indirect calculations of this kind are valueless in the absence of a direct tissue analysis; the fixation of ammonia and of amino acids by the liver is an established fact.

Matthews and Nelson [1914] devised a method for determining the exact character of the metabolic changes taking place in muscle tissue. They removed practically all the viscera with the exception of the kidneys and about 20 to 25 g. of liver in decerebrate dogs, and then injected continuously into the veins a diuretic salt solution to wash all the nitrogen out of the tissues and to keep up an even flow of urine; the "zero" urine thus obtained had a very low nitrogen content and was ammonia-free. 10 g. of erepton containing only traces of ammonia and no urea were then injected in 30 minutes into the muscles. From a comparison of the urine secreted after

the injection with the zero urine the authors claim to have obtained positive evidence that the amino acid mixture injected is broken down and appears in the urine largely as ammonia and urea. The data are very scanty however and the results are not concordant, *e.g.* in dog 3, Table II, the urea is actually diminished after the injection of erepton, and in dog 4 the increase is trivial.

Marshall and Davies [1914] showed that the concentration of urea in the blood and tissues was approximately the same both normally and after the injection of large quantities of urea. The following figures may be quoted:

	Normal dog	After repeated injections of ascending doses	After injection of 5 g. urea in 7 minutes
Blood	28 mg. 27 „	1065 mg.	—
Blood serum	29 „ 29 „	1034 „	493 mg.
Liver	32 „ 23 „	930 „	354 „
Muscle	25 „ 25 „	914 „	377 „

According to these workers the tissues take up with great regularity about 90 per cent. of the amount of urea injected within three minutes after the injection, whether the concentration in the body be high or low. Diffusion of the urea was practically instantaneous and was complete in all parts of the body in 15 minutes. The rate of elimination by the kidneys was very rapid and rose to 16 g. per kilo. body weight or higher per day; when the excretion of urea was prevented the entire amount was stored in the body. No evidence was found of the conversion of urea into any other substance.

McLean and Selling [1914] give some interesting data regarding the urea and non-protein nitrogen in human blood; they found that the concentration of these two forms of nitrogen was not constant in normal individuals but fluctuated from hour to hour and varied within wide limits owing to such factors as diet and the amount of fluid ingested. The figures for the urea nitrogen in their tables range from 10 to 23 mg. per 100 cc. giving a variation of $\pm$ 35 per cent. (*i.e.* the total variation is expressed as a percentage of the mean of the two extreme values), the figures for the non-protein nitrogen vary from 23 to 36 mg. per 100 cc.

Paul György and Edgard Zunz [1915] have recently studied the amino acid content of the blood of normal dogs using the van Slyke method. This value, viz. 4 to 5 mg. per 100 cc., proved remarkably constant, it was not affected by a meal of potato puree but after a meat meal (25 g. meat per

kilo. body weight) the average rose to 11·9 mg. A comparison of the whole blood with the blood plasma showed that this increase was more marked in the case of the blood corpuscles than of the plasma.

Jansen [1915] as a result of perfusing the livers of 26 cats and dogs with blood of varying dilution to which, with two exceptions, additions of leucine, glycocoll, alanine, ammonium carbonate or erepton were made, came to the conclusion that, contrary to the opinion of Fiske and Karsner, the liver played an important part in the formation of urea from amino acids.

In considering the significance of the nitrogen retention which undoubtedly occurs when ammonium salts are introduced into the dietary, the rôle of ammonia in maintaining the normal neutrality of the tissues must not be forgotten. The effect of alkali on autolysis in vitro is to retard the process, and acid on the other hand has an accelerating action. Schryver [1906] obtained practically the same results in the living animal and he believed that the rôle of ammonia in retarding the activity of the proteolytic enzymes of the tissues was a very important one. That large amounts of acid can be introduced into the body by the use of certain foods with a preponderance of acid ash is evident from the work of Shermann and Gettler [1912]; these observers were able to introduce the equivalent of 21·7 cc. and 32·7 cc. N. acid per day on two occasions respectively by substituting rice for potato in a man's diet. The extent to which the acid excreted in the urine is neutralised by the ammonia present in the body is still the subject of conflicting views; in the experiment just mentioned Shermann and Gettler found that the ammonia excreted only accounted for one-quarter to one-half of the acid excreted. Janney [1911] claims that by giving large doses of alkali he was practically able to banish ammonia from the urine.

Lawrence Henderson [1911] finds that the alkalinity of the urine is always lower than that of the blood, and the amount of alkali which must be added to the urine to equalise the two represents the amount of alkali saved to the body. Under normal circumstances he states that the two factors, the physico-chemical separation of acid and ammonia secretion, are probably of the same order of magnitude and are strictly additive. Gammeltoft [1911] turned his attention to the relation of ammonia to the acid secretion of the stomach and continued the investigations of previous workers. Corresponding with the secretion of an acid juice after a meal he found a diminution in the excretion of ammonia in the urine, while following on the subsequent pouring out of an alkaline juice the ammonia excretion rose again.

In view of these facts it is evident that ammonia is constantly needed

to replace the amount excreted in the urine. Now as the only source of ammonia under normal conditions is the protein of the food, it follows that whenever protein is wholly withdrawn from the dietary a shortage of ammonia in the body must occur which will become more pronounced the longer the protein-free diet is continued. Under these conditions the introduction of organic ammonium salts would inevitably lead to a retention in order to restore the normal equilibrium in the tissues. The same argument applies when a shortage of other forms of nitrogen, *e.g.* amino acid nitrogen, has been produced by withholding all nitrogen from the food; given such a condition of nitrogen hunger, the introduction of protein itself or of its cleavage products will almost certainly lead to a retention of nitrogen. In all these experiments in which the nitrogen metabolism of the animal is reduced to a very low level by the administration of nitrogen-free diet, whether it be preceded by a fast or not, this consideration must be borne in mind otherwise the results will receive too favourable an interpretation. Attention has been called to the influence of the previous state of nutrition on the course of metabolic experiments by Pfeiffer and Kriske [1911] in their investigation of the effect on sheep of a protein-rich and a protein-poor diet respectively. It was found that the retention of nitrogen continued longer and was greater in amount in the case of those animals which were in the poorest state of nutrition at the outset of the experiment. In the course of this research, which combined tissue analysis with metabolic work, the authors made an important observation which has a direct bearing on the interpretation of metabolic experiments in general; they stated that the nitrogen retention recorded in the metabolism experiments was usually greater in amount than could be accounted for by the subsequent analysis of the tissue on the slaughter of the animal.

From the experiments of Folin and van Slyke it would seem that the tissues have no selective action with regard to the various forms of nitrogen carried to them by the blood stream, but that they take up amounts of amino acid, urea and ammonia which depend chiefly on the concentration of these bodies in the blood, the higher the concentration the greater the probability will be of finding a fixation on the part of the tissues. In the experiments already mentioned in which ammonium salts were fed, the concentration of ammonia in the blood would certainly be increased above the normal immediately after its absorption and such a condition in itself would favour a retention of nitrogen. A consideration of the facts which have been enumerated should be sufficient to show what caution is needed in

the interpretation of the result of metabolic experiments particularly with regard to attributing a retention of nitrogen to protein synthesis.

Turning to the tissues themselves, as we have seen under normal circumstances, a definite percentage of ammonia, amide and amino acid nitrogen is present which can readily be extracted, and although there is a considerable degree of variation in the actual and relative amount of these various substances the tissues never get entirely depleted. It is to be supposed that the amino acids present have been mainly derived from the proteins of the food and have been absorbed as such, while a small proportion has probably come from the catabolic processes of the cells themselves. Some of these amino acids must be destined for constructive and others for degradation purposes, only a very small proportion being normally excreted unchanged in the urine. Possibly the percentage of amino acid normally present in the tissues represents the point of equilibrium between the anabolic and catabolic processes in the cell. The amide has presumably originated in the tissues themselves mainly from the amino acid of the food but in a lesser degree as a degradation product of cell metabolism. The percentage is much lower than that of the amino acid, possibly indicating that it takes part in fewer transactions particularly of a synthetic nature; there is no evidence that it can be utilised for synthetic purposes beyond the feeding experiments previously described, in which urea caused a marked retention of nitrogen, and the outstanding feature of these investigations was the inability of the amide to act as an adequate substitute for protein.

The percentage of ammonia, the simplest form of tissue nitrogen, is again very much smaller than that of the amide. Does this small quantity of ammonia indicate participation in a very small number of metabolic transactions or does it represent the minimum necessary for purposes of neutralisation, *i.e.* to regulate the reaction of the tissues? There is no doubt that ammonia is mainly derived from protein degradation and the evidence of its resynthesis into protein in the body, as we have seen, is certainly not of a nature to suggest that such a process occurs to any extent under normal circumstances.

It will be shown later that there is a higher percentage of amino acid and of amide nitrogen in the liver than in the muscle, the reason for which may be that a larger proportion of the amino acid of the food is metabolised in the former than in the latter.

These three forms of nitrogen are so readily washed out of the tissues that one cannot imagine them to be in chemical union with any of the cell constituents. The same consideration applies to the fixation by the tissues

of these same nitrogenous bodies when introduced directly into the blood stream. They are so easily extracted that the nature of their union with the cell seems much more likely to be physical than chemical. The process of adsorption offers at once the simplest and most feasible explanation of the bond, and the release of the adsorbed substance would immediately follow on lowering the concentration of the solution in the process of extraction. Bayliss [1915] points out that adsorption is a necessary preliminary to many chemical reactions and certainly in the body with its colloidal solutions there must be an infinite number of chemical processes which are initiated in this way.

Composition of Normal Tissues.

When the present investigation was started four years ago the interest was focused on the capacity of the liver to fix and absorb ammonia after its introduction into the blood stream, and on the question of whether the ammonia content of the tissues was affected by different diets. It was hoped by adopting the following method that the same set of experiments would throw light on both points. Previous to the actual experiment the dogs were fed on two diets which differed as widely as possible in their nitrogen content, the one consisting of lard and tapioca and the other of meat with or without an addition of dog biscuit. It was expected that a comparison of the initial tissue analyses in the case of animals fed on the two different diets would furnish data as to the part played by diet in the ammonia content of the tissues and that a comparison of the tissue analyses after the injection would determine the changes in the ammonia content caused by the injection. The actual experiment was carried out under complete ether narcosis; after one or more lobes of the liver had been ligatured off for the initial tissue analysis, the ammonium salt was injected intravenously. The animal was killed by bleeding shortly after the cessation of the injection and the remaining lobes of the liver were then utilised for the determination of the final ammonia content.

After the first few experiments the research began to widen out, muscle tissue was analysed as well as liver, and in addition to the ammonia, total soluble or non-protein nitrogen, amino acid and amide nitrogen were determined with a view to finding out the initial nitrogen distribution in the tissues and the effect produced on it by intravenous injection. An investigation of the disappearance of ammonia from the blood was quickly given up

because of the impossibility of carrying out so many estimations single-handed, and for the same reason an analysis of the intestinal mucosa was only carried out in four of the earlier experiments. A further question arose after the first seven experiments, viz. was there any evidence of protein synthesis after the intravenous injection of ammonia, and this entailed a determination of the total nitrogen of the tissues before and after injection together with an estimation of their water content.

As the initial variations in the nitrogen content of the tissues were found to be very wide, the attempt to determine the influence of diet upon it was abandoned after the fifteenth experiment (dog G); the variations up to this time had no definite relation to diet and were therefore judged to be physiological, and the correctness of this deduction was confirmed by the subsequent work in which the variations were about equally wide although a uniform diet of porridge and milk was employed[1].

For purposes of comparison and control a short series of experiments was carried out to ascertain whether the intravenous injection of physiological salt solution caused any change in the nitrogen content and its distribution or in the water content of the tissues. In the later experiments the effect of injecting glycocoll and urea was also investigated. Unfortunately the unavoidable interruption of the research led to its extension over more than two years, and as the later experiments were planned in the light of the earlier work, they naturally furnished on the whole more concordant results and required fewer repetitions. In dealing with the methods employed it is only necessary to describe the procedure in the later experiments as any modifications introduced have only been slight. The results of the experiments are all tabulated in Tables I–XV.

Technique and methods.

After a preliminary hypodermic injection of morphia the animal was anaesthetised with chloroform followed by ether. The jugular vein was first exposed and ligatures were placed in position, the left common iliac artery and vein were then ligatured to facilitate amputation of the thigh, which was performed as high up as possible in order to furnish the maximum amount of muscle tissue for analysis; the bleeding was completely controlled by including all the muscles above the seat of amputation in three interlaced tape ligatures. If the bladder contained any urine it was emptied by direct

[1] This does not imply that diet has no influence on the composition of the tissues but that no appreciable change could be attributed to the diet in these particular experiments.

pressure or failing this by an incision, which was then closed by a purse-string suture. Two or even three lobes of the liver were then ligatured with tape and removed, and immediately after this the cannula was inserted into the jugular vein and the intravenous injection was started. The solution was warmed before being placed in the burette and was run in continuously in the case of the urea and glycocoll but the injection of the ammonia had to be frequently interrupted because of its toxic effect. The animal was killed by bleeding about ten minutes after the cessation of the injection; a few exceptions in the case of some of the ammonia experiments were due to the death of the animal from ammonia poisoning during the injection[1]. After the death of the animal the rest of the liver was at once removed and the other thigh was amputated. If the bladder contained any urine it was ligatured off and removed to prevent any loss of the contents. The blood was carefully and thoroughly squeezed out of each lobe of the liver, immediately after its removal, by firm pressure with the fingers; three small portions from about 0·2 to 0·3 g. of tissue were quickly excised and placed in weighing bottles of known weight, the latter were immediately replaced in the desiccator and were weighed as soon as possible. When practicable 100 g. of liver were used for the determination of the non-protein nitrogen but sometimes a much smaller quantity only was available, viz. 50 to 60 g. After weighing, the tissue was plunged into 100 cc. of acidified boiling water and after being cut up in the fluid into small pieces with scissors in order to ensure complete coagulation of the protein it was boiled for five minutes. (This particular modification of the earlier technique, in which the tissue was boiled in acidified water after being ground up with glass, was adopted from the work of van Slyke.) The muscle tissue was treated in the same way.

The further treatment of the tissue set aside for the determination of the non-protein nitrogen and its constituents was as follows: the liquid was poured off into a porcelain basin and the tissue was pounded with ground glass in a mortar and was then transferred to the liquid in the basin with an additional 100 cc. of water, the whole was then boiled with constant stirring to prevent bumping. After cooling the precipitate and filtrate were transferred to a 500 cc. cylinder and the volume was made up to 400 cc.

[1] One of the five dogs destined for the injection of normal saline solution died before the injection started apparently from an overdose of the anaesthetic, the tissues were worked up in the usual way however, duplicate samples of liver and muscle being taken for the determination of the soluble nitrogen and its constituents. Although both values have been given in the tables only the figures in the column of initial values have been included in the calculation of the various averages relating to the whole series or to the particular group of experiments.

Aliquot portions of the filtrate were taken for the various analyses, 25 cc. for the non-protein nitrogen and 50 cc. for the ammonia, amide and amino nitrogen. Duplicate analyses of the ammonia and amide nitrogen could not be carried out owing to the number of determinations necessary, but a sufficient check was established by the repetition of the experiments. The analytical methods employed were the same as those used in the previous work [Bostock, 1912, 1] on the nitrogen distribution in autolysis and were as follows: non-protein nitrogen by the Kjeldahl method, ammonia by the Kruger-Reich-Schittenhelm method, amide by a modification of the Benedict-Gephart autoclave method and amino acid by the Henriques-Sörensen method, the initial neutralisation being carried out with litmus as indicator.

The total nitrogen was at first estimated on the moist tissue in duplicate, in all the later work, however, it was estimated in triplicate on the dried tissue; the same samples having been previously weighed in the moist condition served also for the determination of the percentage of dried matter in the fresh tissue, drying being carried out at a temperature of from 100° to 103°.

There is no record of any observations dealing primarily with variations in the composition of the normal tissues and little attention has been drawn to the fact that very considerable differences do occur, although the initial tissue analysis in certain types of experiment furnishes some data on the subject. Emmet and Carroll [1911] however have pointed out some most interesting facts with regard to the physical constants of fat; there was no change produced in the fat of an animal by feeding it with different amounts of protein but great differences were found in the fat of the same animal in different regions of the body, *e.g.* the melting point was 34·1° for the back fat and 45·7° for the kidney fat. Van Slyke [1913, 2] drew attention to the fact that the amino acid content of the gracilis muscle was normally about 10 to 20 mg. less per 100 g. than that of the triceps, and elsewhere [1913, 1] he stated that when different samples of an organ such as the liver were taken as duplicate samples or when symmetrically placed organs such as the right and left kidney gracilis were used, a variation up to 10 per cent. of the amount of amino nitrogen determined must be expected. The following instances may be quoted: lobe (1) 34 mg., lobe (2) 29 mg. [van Slyke and Meyer, 1913, 1], and again, lobe (1) 59 mg., lobe (2) 55 mg., lobe (3) 64 mg. [van Slyke and Meyer, 1913, 3].

That variations exist in the non-protein nitrogen of the blood in different individuals of the same species may be seen from an examination of Folin's

figures; in the first series of experiments [Folin and Denis, 1912, 1] these range from 30 to 40 mg. per 100 cc. of portal blood, while in a later series [Folin and Denis, 1912, 3] the extent of the variation is even greater, the limits per 100 cc. of blood being 29 and 67 mg.; the minimum values it may be mentioned in the last named experiments are unmistakably due to the diet.

The nitrogen of the tissues is of course mainly in the form of coagulable or protein nitrogen but a small percentage can be washed out of the tissue, which is neither coagulable by heat nor affected by the ordinary precipitants of protein. The amount of this soluble nitrogen varies slightly according to the method used, in the case of that adopted in the present research it amounts to about 11 per cent. of the total nitrogen. The main constituents of the soluble or non-protein nitrogen are the amino acid, amide and ammonia nitrogen and together they constitute about 60 per cent. of the soluble nitrogen.

The term *nitrogen distribution* in this paper is used to designate the actual and relative amounts of these different forms of nitrogen, and the present series of experiments will furnish data regarding the particular arrangement existing in different tissues and the changes produced by the intravenous introduction of various forms of nitrogen. The variations which occur in different individuals in the amount of the various nitrogenous bodies and in the relations existing between some of these may be conveniently described under the heading of *physiological variation*, and it will be necessary to consider its limits before proceeding to the study of the effects produced on the nitrogen distribution in the tissues by the introduction of different types of nitrogen into the blood stream.

The difference between the maximum and minimum values in a given series of figures furnishes the total variation for the series and if this figure be halved the total $\pm$ variation is obtained; the significance of the variation can only be accurately gauged, however, by expressing it as a percentage of the average value for the whole series of figures under consideration and such a procedure also renders it available for purposes of comparison. This method of treating the total $\pm$ variation has been adopted in the present paper. For example, the maximum and minimum values for the series of figures 4, 3 and 2 are 4 and 2 respectively, the total variation is 2 and the total $\pm$ variation is $\pm$ 1; the mean of the series is 3 and therefore the percentage variation is $\pm$ 33·3 per cent. $\left(\frac{1 \times 100}{3}\right)$.

In the case of figures taken from the work of other investigators, the mean of the maximum and the minimum values has been substituted for

the mean of the whole series as it was more readily arrived at and did not seriously affect the resulting value.

The physiological variation in the normal tissues will be considered under the following headings:

1. Percentage of dried material in 100 g. fresh tissue.
2. Total nitrogen in 100 g. fresh and dried tissue.
3. Total insoluble or protein nitrogen in 100 g. fresh and dried tissue.
4. Total soluble or non-protein nitrogen in 100 g. fresh tissue.
5. Relationship of the total protein and non-protein nitrogen to the total nitrogen.
6. Amino acid nitrogen in 100 g. fresh tissue.
7. Amide nitrogen in 100 g. fresh tissue.
8. Ammonia nitrogen in 100 g. fresh tissue.

1. *Physiological variation in the percentage of dried tissue in* 100 *g. fresh tissue.*

It is evident that the percentage of moisture in the tissues will affect the figures for the nitrogen content of the fresh tissue and it might conceivably be responsible for the variations in the latter. It will be seen from Table I that there are considerable differences in the percentage of dried matter in the fresh tissue of both liver and muscle in different individuals. The limits for the liver are 28·4 per cent. and 21·9 per cent. giving a total variation of ± 3·2 or of ± 12·1 per cent., which is less than the percentage variation in the values for every variety of nitrogen in the fresh liver, and therefore the amount of moisture cannot be the determining factor in variations of the nitrogen content. The average value for the whole series is 26·64 per cent. and may be compared with the average for Grund's whole series which is 25·5 per cent. In the case of the muscle the limits are 28·4 per cent. and 22·3 per cent. giving the same percentage variation of ± 12·1 per cent. as in the liver; here again the variation in the moisture is smaller than the variation in any of the forms of nitrogen present in the fresh muscle. The average value for the muscle is 24·8 per cent. and is slightly higher than Grund's average, which works out at 23·9 per cent. It may be mentioned that it by no means always follows that a high percentage of moisture in the liver is associated with the same condition in the muscle; in about 47 per cent. of the cases the value is above the average in the one and below it in the other tissue and neither the maximum nor minimum value for the liver is

associated with the corresponding maximum or minimum value in the muscle.

TABLE I.

Giving percentage of dried matter in 100 g. fresh tissue before and after injection.

Experiments		Liver		Muscle	
		Before	After	Before	After
Early ammonia	*	28·41	25·54	24·59	24·57
,,	A	26·1	26·2	28·4	28·4
,,	B	26·3	25·9	27·7	25·9
,,	C	27·72	25·95	26·25	25·55
,,	D	26·92	24·33	23·8	23·78
,,	E	27·25	26·98	24·32	23·11
,,	F	27·71	23·87	24·46	23·68
,,	G	27·68	25·92	26·23	25·17
Last ammonia	I	21·9	21·7	23·1	23·5
,,	II	26·7	24·5	22·3	22·6
Sodium chloride	I	27·71	28·43	26·66	26·67
,,	II	27·97	26·23	26·04	26·88
,,	III	28·07	24·68	24·49	25·55
,,	IV	26·38	21·77	24·89	25·14
,,	V	26·82	24·61	25·06	24·73
Glycocoll	I	24·73	22·14	23·69	23·49
,,	II	24·38	24·57	24·61	23·33
,,	III	26·94	22·15	24·39	24·11
,,	IV	26·8	24·9	24·17	24·7
,,	V	27·61	25·40	25·11	25·66
,,	VI	26·64	24·34	24·46	24·36
Urea	I	26·5	27·1	22·8	23·5
,,	II	25·5	25·7	25·05	24·62
Average for whole series		26·64	25·05	24·89	24·72

2. *Total nitrogen calculated on the fresh tissue.*

These figures will be found in Table II. The average value for 100 g. liver is 3·064 g. and there is a total variation of ± 0·443, or of ± 14·4 per cent. The average value for the muscle is slightly higher and is 3·397 g., with a total variation of ± 0·516 g. or of ± 15·1 per cent. Neither the maximum nor the minimum values for the liver are found in conjunction with the maximum or minimum values for the muscle, and the lack of any close correspondence between the relative amounts of the total nitrogen of the two tissues is borne out by the fact that in 38 per cent. of the experiments a high value in the one tissue is accompanied by a low value in the other. Buglia and Constantino [1913] give 3·240 g. and 3·390 g. for the total nitrogen of the muscle of two fed dogs; these figures coincide closely with

the average value given above. Diesselhorst's figures [1911] in the case of two dogs are as follows:

Rice diet: Dog 1, 3·08 g. ... Dog 2, 3·21 g.
Meat diet: Dog 1, 3·27 g. ... Dog 2, 3·46 g.

TABLE II.

Giving the total nitrogen in g. in 100 g. fresh and dried tissue before and after injection.

Experiments		Fresh liver		Dried liver		Fresh muscle		Dried muscle	
		Before	After	Before	After	Before	After	Before	After
Early ammonia	*	3·192	3·287	11·23	12·88	3·599	3·631	14·63	14·77
,,	A	2·990	2·890	11·45	11·03	4·081	3·318	14·36	11·68
,,	B	2·891	2·289	10·99	8·84	3·346	3·069	12·08	11·85
,,	C	2·590	2·947	9·34	11·35	3·365	3·328	12·81	13·02
,,	D	3·165	2·029	11·75	8·33	3·302	3·252	13·87	13·67
,,	E	3·058	3·370	11·22	12·49	3·269	3·150	13·44	13·63
,,	F	2·793	2·933	10·08	12·28	3·328	3·188	13·60	13·46
,,	G	2·591	2·950	9·36	11·38	3·366	3·328	12·83	13·22
Last ammonia	I	3·128	3·011	12·42	13·25	3·247	3·351	13·48	14·04
,,	II	2·918	3·037	10·92	12·39	3·048	3·112	13·62	13·69
Sodium chloride	I	3·251	3·489	11·73	12·27	3·482	3·471	13·05	13·01
,,	II	2·697	2·859	9·64	10·90	—	—	—	—
,,	III	3·009	2·952	10·71	11·95	3·277	3·312	13·37	12·96
,,	IV	3·185	2·730	12·02	12·52	3·492	3·485	14·02	13·90
,,	V	3·043	3·022	11·59	12·42	3·409	3·398	13·60	13·79
Glycocoll	I	3·070	2·970	12·41	13·41	3·290	3·352	13·88	14·27
,,	II	3·202	3·333	13·13	13·56	3·494	3·606	14·19	15·45
,,	III	3·107	2·746	11·53	12·39	3·448	3·579	14·13	14·84
,,	IV	3·467	3·209	12·93	12·87	3·381	4·131	14·19	16·67
,,	V	2·960	3·046	10·71	11·96	3·504	3·597	13·91	14·01
,,	VI	3·476	3·299	13·04	13·55	—	—	—	—
Urea	I	3·419	3·578	12·88	13·17	3·213	3·326	14·04	14·13
,,	II	3·271	3·322	12·82	12·88	3·409	3·426	13·60	13·91
Average for whole series		3·064	3·012	11·47	12·07	3·397	3·396	13·65	13·80

Calculated on 100 g. dried tissue the average value for the liver is 11·47 g. with a total variation of ± 1·89 g. or of ± 16·4 per cent. The minimum value coincides with the minimum value for the fresh tissue and Table III shows that there is a general correspondence between the values for the moist and dried tissue in the different groups of experiments, a high or low value in the one being accompanied by a correspondingly high or low value in the other with the exception of the last ammonia group. The average value for muscle is 13·65 g. with a total variation of ± 1·275 g. or of ± 9·3 per cent.

Buglia and Constantino's values for the dried muscle are 14·08 g. and 13·87 g. and those of Diesselhorst are:

Rice diet: Dog 1, 12·82 g. ... Dog 2, 11·44 g.
Meat diet: Dog 1, 11·54 g. ... Dog 2, 12·88 g.

The maximum and minimum values for the dried muscle do not coincide with the corresponding values for the moist muscle and this lack of concordance is evident in two out of the five groups of experiments in Table III, where a high value in the dried tissue is associated with a low value in the fresh and *vice versa*.

TABLE III.

Average value in g. for the different groups of experiments of the total nitrogen *in* 100 *g. moist and dried tissue before and after injection.*

Experiments	Fresh liver		Dried liver		Fresh muscle		Dried muscle	
	Before	After	Before	After	Before	After	Before	After
Early ammonia ...	2·908	2·836	10·67	11·07	3·407	3·283	13·40	13·16
Last ammonia ...	3·023	3·024	11·67	12·82	3·147	3·231	13·55	13·86
Sodium chloride ...	3·000	3·080	11·41	11·88	3·389	3·393	13·34	13·25
Glycocoll ...	3·214	3·100	12·29	12·95	3·423	3·653	14·06	15·04
Urea ...	3·345	3·450	12·85	13·0	3·311	3·376	13·82	14·02
Mean value for the whole series	3·064	3·012	11·47	12·07	3·397	3·396	13·65	13·80

There is a rather closer agreement between the relative amounts of the total nitrogen of the liver and muscle than was found in the case of the total nitrogen calculated on the moist tissue, for in 33 per cent. only of the experiments is a relatively high value in the one tissue associated with a relatively low value in the other. In comparing the percentage variation, it will be seen that it is greater in the case of the dried liver than in that of the fresh organ but the reverse is true of the muscle, showing that in the latter case the water content of the tissue is responsible for some of the variation found in the analysis of the fresh material.

3. *The total insoluble or protein nitrogen.*

The figures for the insoluble nitrogen are obtained by subtracting the total soluble nitrogen from the total nitrogen, the average value for 100 g. liver is 2·772 g. and the total variation is ± 0·398 g. or ± 14·3 per cent. The average value for the muscle is 3·018 g. with a total variation of ± 0·528 g. or ± 17·4 per cent. The maximum value for the one tissue does not coincide with the maximum for the other and the same holds good

of the minimum values; in 55 per cent. of the experiments the value is above or below the average respectively in both liver and muscle while in the remaining 45 per cent. a high value in the one is associated with a low value in the other.

TABLE IV.

Total insoluble or protein nitrogen in g. in 100 g. moist and dried tissue respectively before and after injection.

		Fresh tissue				Dried tissue			
		Liver		Muscle		Liver		Muscle	
Experiments		Before	After	Before	After	Before	After	Before	After
Early ammonia	*	2·809	2·934	3·239	3·264	9·88	11·5	13·7	13·2
,,	A	2·778	2·653	3·727	2·971	10·63	10·25	13·08	10·4
,,	B	2·656	2·037	2·963	2·734	10·48	7·8	10·6	10·6
,,	C	2·374	2·681	2·973	2·943	8·70	10·2	11·3	11·5
,,	E	2·833	3·145	2·905	2·789	10·48	11·5	11·8	12·0
,,	F	2·577	2·695	2·986	2·845	9·35	11·2	12·2	12·0
,,	G	2·425	2·759	3·052	3·003	9·52	10·6	11·6	11·9
Last ammonia	I	2·885	2·765	2·852	2·948	13·80	12·7	12·4	12·5
,,	II	2·554	2·667	2·670	2·726	9·56	10·8	11·9	12·0
Sodium chloride	I	2·965	3·183	3·100	3·088	10·7	11·1	11·8	11·2
,,	II	2·426	2·581	—	—	8·7	9·8	—	—
,,	III	2·654	2·571	2·951	2·967	9·44	10·4	11·8	11·6
,,	IV	2·830	2·409	3·085	3·102	10·76	11·0	12·4	12·0
,,	V	2·774	2·726	3·061	3·055	10·4	11·0	12·2	12·1
Glycocoll	I	2·677	2·615	2·944	2·988	10·8	11·7	12·3	12·7
,,	II	2·939	3·020	3·110	3·229	12·0	12·2	12·6	12·9
,,	III	2·742	2·422	3·036	3·159	10·6	9·7	12·3	12·8
,,	IV	3·147	2·830	3·011	3·738	11·75	11·3	12·4	15·1
,,	V	2·637	2·665	3·132	3·202	10·36	10·4	12·3	12·5
,,	VI	3·171	2·937	—	—	12·33	12·0	—	—
Urea	I	3·085	3·191	2·803	2·855	11·62	11·7	12·3	12·1
,,	II	2·980	3·005	3·016	2·987	11·7	11·2	11·9	12·1
Average for whole series		2·772	2·701	3·018	3·013	10·4	10·8	12·0	12·1

The average value for the liver is smaller than for the muscle and in only 15 per cent. of the experiments is the reverse condition found. Grund's [1910] figures for the protein nitrogen are slightly lower than mine; they vary between 2·2 g. and 2·5 g. for the liver and between 2·5 g. and 2·8 g. for the muscle in the case of his six fed dogs.

It is of interest to note that the percentage variation of the insoluble nitrogen is much smaller than that of the soluble or non-protein nitrogen. Calculated on the dried tissue the average for the liver is 10·4 g. with a variation of ± 2·55 g. or ± 24·5 per cent. For the muscle the average is 12·0 g. with

a variation of ± 1·55 g. or ± 12·9 per cent. In the case of the liver the variation on the dried weight is considerably greater than on the fresh tissue, which proves conclusively that the main source of the variation does not lie in the percentage of moisture in the tissue. There is only a single exception, namely Experiment ammonia I, to the rule that the protein nitrogen of the dried liver is smaller than that of the muscle.

4. *Total soluble or non-protein nitrogen.*

The figures for the total soluble nitrogen of the fresh tissue will be found in Table V, and glancing at them one is at once struck by the very varying amounts present in the livers of different individuals; while the average for the whole series is 292 mg. in 100 g. fresh tissue there is a total variation of ± 144·5 mg. or of ± 49·4 per cent. The average for the muscle is 379 mg. with a total variation of ± 86·5 mg. or of ± 21·6 per cent. Buglia and Constantino [1913] found almost identical values for the non-protein nitrogen in the case of two dogs, the actual figures being 370 mg. and 390 mg. in 100 g. muscle. Folin's figures [Folin and Denis, 1912, 3] for muscle show a variation of ± 31 per cent. but in this case the diet is certainly responsible for the minimum values. The figures of Fiske and Sumner [1914], who used the methods of Folin, show a total variation of ± 55·5 mg. or of ± 21 per cent. The actual values for the non-protein nitrogen are lower than in my experiments, due probably to the different species of animal and the different method employed. Although in the present research the highest values for the liver and muscle occurred in the same dog and the lowest values were similarly found in one and the same animal, a relatively high nitrogen content in one tissue was by no means always accompanied by high content in the other, for in 50 per cent. of the cases a value above the average in one tissue is associated with a value below the average in the other.

5. *Relationship of the protein and non-protein nitrogen to the total nitrogen.*

The relation of the protein and non-protein nitrogen to the total nitrogen will be found in Tables VI and VII expressed as a percentage of the latter. The average ratio of the non-protein nitrogen to the total nitrogen in the liver for the whole series is 9·5 per cent. with a total variation of ± 3·19 or ± 33·5 per cent., the average for the muscle is 11·1 per cent. with a total variation of ± 2·01 or of ± 18·1 per cent.; in this relation therefore the limits of variation are also narrower in the case of the muscle than of the liver. Although the average value is rather smaller in the case of the liver than of

TABLE V.

Giving the non-protein, amino acid, amide and ammonia nitrogen in mg. per 100 g. fresh tissue before and after injection.

		Liver								Muscle							
		Total soluble N		Amino acid N		Amide N		Ammonia N		Total soluble N		Amino acid N		Amide N		Ammonia N	
Experiments		Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Early ammonia	I	—	—	—	—	—	—	11·0	14·9	—	—	—	—	—	—	—	—
,,	II	—	240	—	—	—	—	14·3	27·5	—	—	—	—	—	—	—	—
,,	III	313	358	—	—	48·7	52·9	10·0	11·4	436	403	—	—	46·4	44·7	13·4	14·0
,,	IV	261	239	—	—	24·6	21·8	7·0	20·1	324	347	—	—	23·5	27·3	10·0	11·2
,,	V	257	263	—	—	27·4	38·0	10·6	8·9	397	380	—	—	32·7	31·9	12·0	12·6
,,	VII	445	414	72·9	71·3	48·6	58·2	9·6	11·6	487	456	55·4	52·0	34·9	37·0	9·8	12·8
,,	VIII	350	327	52·0	57·7	57·2	58·4	3·2	5·6	368	385	39·7	33·4	43·3	41·9	8·4	13·1
,,	*	383	359	—	—	—	—	—	—	360	367	—	—	—	—	—	—
,,	A	212	237	78·3	72·4	29·4	37·2	8·3	11·4	354	347	75·3	68·6	33·1	29·6	10·8	8·4
,,	B	235	252	70·7	73·0	27·7	39·1	5·6	9·5	383	335	74·0	64·8	31·6	31·3	9·2	14·2
,,	C	216	266	54·1	62·2	33·4	37·6	5·6	21·7	392	385	63·5	69·5	36·6	37·8	9·2	10·9
,,	D	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
,,	E	225	225	58·0	47·9	33·5	40·9	7·0	17·0	364	361	66·0	56·6	35·0	37·0	9·3	17·6
,,	F	216	238	**76·0**	**72·4** = amino acid + ammonia N					342	343	**91·0**	**90·2** = amino acid + ammonia N				
,,	G	166	191	—	—	—	—	—	—	314	325	—	—	—	—	—	—
Last ammonia	I	243	246	76·1	65·2	37·0	39·8	5·6	18·9	395	403	89·8	87·5	36·9	43·1	9·5	14·0
,,	II	364	370	73·2	67·1	50·7	62·8	5·2	26·4	378	386	85·8	84·2	53·1	57·8	12·1	14·9
,,	III	236	240	63·3	42·0	43·2	40·1	5·0	37·5	379	407	72·1	76·8	32·2	42·7	18·9	20·3
Sodium chloride	I	286	296	72·6	71·8	—	—	10·7	12·9	382	383	—	—	—	—	14·9	14·9
,,	II	271	278	—	—	—	—	4·4	7·8	378	371	71·2	70·9	—	—	7·8	8·9
,,	III	355	381	100·7	107	42·6	43·1	13·3	10·1	326	345	81·8	77·4	36·1	37·3	9·9	9·9
,,	IV	355	321	98·8	80·5	—	—	7·0	7·0	407	383	73·3	80·9	38·0	34·7	9·5	10·0
,,	V	269	296	78·6	84·2	41·4	41·4	6·7	6·7	348	343	83·8	82·0	30·1	29·4	9·8	9·8
Glycocoll	I	393	355	**74·2**	**110·6** = amino acid + ammonia N				—	346	364	—	—	—	—	—	—
,,	II	263	313	67·0	80·8	—	—	13·3	22·5	384	377	—	—	—	—	—	—
,,	III	355	324	96·5	116·6	—	—	5·6	12·8	412	420	82·4	82·3	—	—	10·6	10·0
,,	IV	320	407 351	83·7	156·2 136·3	58·2	64·3 58·8	10·0	17·3 16·2	370	393	89·6	94·7	62·7	63·9	8·4	8·9
,,	V	323	354 408	77·2	138·9 136·3	44·1	51·5 51·3	10·2	10·2 12·8	372	395	96·7	104·2	38·1	40·5	8·2	9·1
,,	VI	305	345 389	68·8	139·7 142·7	50·0	45·3 50·1	7·1	16·6 16·6	399	430	84·1	103·0	43·1	47·5	11·7	11·7
Urea	I	334	387	87·1	76·5	56·6	138·1	9·0	11·6	410	471	85·2	84·9	47·6	98·7	11·9	12·6
,,	II	291	317	72·1	68·4	44·9	115·1	8·6	9·4	393	439	87·8	89·0	44·1	93·0	12·4	12·7
Average for the whole series		292	311	76·3	93·1	41·2	53·6	8·0	15·5	379	383	78·7	79·3	38·6	46·3	10·8	12·3

The figures of Experiment ammonia VIII referring to the goat have not been included in the various averages.

the muscle this condition is reversed in 20 per cent. of the individual experiments. A relatively high or low value in the liver is associated with a correspondingly high or low value in the muscle in 55 per cent. of the experiments.

The average ratio of the protein nitrogen to the total nitrogen in the liver is 90·5 per cent. with the same total variation as in the case of the ratio

TABLE VI.

Giving insoluble or protein nitrogen as a percentage of the total nitrogen before and after injection.

Experiments		Liver		Muscle	
		Before	After	Before	After
Early ammonia	*	88·0	89·26	90·0	90·0
,,	A	92·90	91·80	92	89·6
,,	B	91·88	89·00	88·6	89·0
,,	C	91·67	90·97	88·4	88·4
,,	D	—	—	—	—
,,	E	92·62	93·32	88·8	88·6
,,	F	92·27	91·90	89·7	89·2
,,	G	93·60	93·53	90·6	90·2
Last ammonia	I	92·22	91·82	87·9	88
,,	II	87·52	87·82	87·6	87·6
Sodium chloride	I	91·20	91·24	89	89
,,	II	89·95	90·28	—	—
,,	III	88·20	87·10	90·1	89·6
,,	IV	88·85	88·23	88·3	89·1
,,	V	91·16	90·20	89·8	89·5
Glycocoll	I	87·19	88·04	89·4	89
,,	II	91·79	90·69	89·0	89·6
,,	III	88·25	88·18	88·1	88·3
,,	IV	90·78	88·18	89	90·5
,,	V	89·08	87·50	89·4	89·1
,,	VI	91·22	89·03	—	—
Urea	I	90·23	89·18	87·3	87·9
,,	II	91·12	90·46	88·5	87·2
Average for whole series		90·5	89·7	88·9	88·8

of non-protein to total nitrogen, which however only amounts to ± 3·5 per cent., when expressed as the percentage variation; this shows in a striking fashion how much more liable to variation is the non-protein nitrogen than the protein nitrogen. The average value for the muscle is 88·9 per cent. with a total variation of ± 2·01 or of ± 2·2 per cent.

6. *The amino acid nitrogen* (see Tables V and XIII).

The average value for 100 g. fresh liver is 76·3 mg., the total variation being ± 23·3 mg. or ± 30·5 per cent.; a study of the initial values

in van Slyke's [van Slyke and Meyer, 1913, 1] experiments reveals a total variation of ± 13·5 mg. or of ± 31 per cent. (calculated as a percentage of the mean (42·5 mg.) of the maximum and miminum values). In one case the amino acid content of two different lobes of the same liver gave a total

TABLE VII.

Giving total soluble or non-protein nitrogen as a percentage of the total nitrogen before and after injection.

Experiments		Liver		Muscle	
		Before	After	Before	After
Early ammonia	*	12·01	10·92	10·02	10·11
,,	A	7·11	8·23	8·67	10·46
,,	B	8·13	11·01	11·44	10·91
,,	C	8·34	12·42	11·65	11·57
,,	D	—	—	—	—
,,	E	7·35	6·67	11·13	11·45
,,	F	7·74	8·13	10·3	10·77
,,	G	6·42	6·47	9·34	9·79
Last ammonia	I	7·76	8·16	12·16	12·04
,,	II	11·91	11·63	12·4	12·41
Sodium chloride	I	8·79	8·48	10·97	11·03
,,	II	10·05	9·72	—	—
,,	III	11·83	12·9	9·96	10·41
,,	IV	11·15	11·76	11·65	10·9
,,	V	8·83	9·79	10·2	10·5
Glycocoll	I	12·80	11·9	10·51	10·85
,,	II	8·21	9·39	11·0	10·4
,,	III	11·42	11·79	11·94	11·73
,,	IV	9·22	10·93 12·7	10·94	9·51
,,	V	10·93	11·62 13·41	10·61	10·99
,,	VI	8·77	10·45 11·79	—	—
Urea	I	9·76	10·8	12·72	14·16
,,	II	8·89	9·55	11·53	12·81
Average for whole series		9·5	10·3	11·1	11·2

variation of 8 per cent. It will be seen that the Sörensen method in my hands gave a rather higher figure than van Slyke found by his method.

The average value for the muscle is 78·7 mg. with a total variation of ± 20·6 mg. or of ± 26·1 per cent. In the case of the gracilis, van Slyke's figures show a total variation of ± 15·5 mg. or of ± 30 per cent.; the extremely interesting fact that the amino acid content of the triceps is usually 10 to 20 mg. higher than that of the gracilis has already been referred to. Buglia

and Constantino using the Sörensen method found an amino acid content in the muscle of two fed dogs of 84 mg. and 77 mg. respectively.

In 64 per cent. of the experiments a relatively high or low value in the liver is associated with a correspondingly high or low value in the muscle but neither maximum nor minimum values occur in the same animal. Although the average value for the muscle is slightly higher than that of the liver this condition is reversed in 35 per cent. of the experiments and a similar variability is present in van Slyke's experiments, the amino acid content of the liver being sometimes higher and at other times lower than that of the muscle.

In comparing the values of the amino acid nitrogen with those of the soluble nitrogen, 73 per cent. of the experiments show a relatively high or low value for both forms of nitrogen in the liver although neither the maximum nor the minimum values occur in the same dog; 47 per cent. of the experiments show a relatively high or low value for both forms of nitrogen in the case of the muscle.

7. *Amide nitrogen* (see Tables V and XIII).

The average value for the amide nitrogen in 100 g. fresh liver is 41·2 mg. and the total variation is ± 16·8 mg. or ± 40·7 per cent.; for the muscle the average is slightly smaller, namely, 38·6 mg. with a total variation of ± 19·6 mg. or ± 50·7 per cent. The maximum values for both tissues occur in the same dog and the same holds good for the minimum values; in only 28 per cent. of the experiments is a relatively high value in the one tissue associated with a relatively low value in the other. In Folin's first paper there is a total variation in the urea values for the muscle of ± 11·5 mg. or of ± 29 per cent. (calculated as a percentage of the mean of the maximum and minimum values), in his third paper the percentage amounts to ± 44 per cent. but here also as in the case of the non-protein nitrogen the influence of diet is unmistakable, the minimum values occurring in cats fed on a rice and cream diet. In Fiske and Sumner's [1914] experiments the urea values for the muscle range from 14 mg. to 82 mg. which gives a total variation of ± 70 per cent.

Although the average figure for the liver is slightly higher than for the muscle the reverse condition is present in 39 per cent. of the experiments. In the liver there is quite a close general correspondence between the values for the total soluble nitrogen and the amide nitrogen; although neither maximum nor minimum values are found in the same animal, in 83 per cent. of the cases a relatively high value for the one form of nitrogen is associated

with a correspondingly high value for the other form and *vice versa*. In the muscle on the other hand such a correspondence is only present in 55 per cent. of the experiments.

8. *Ammonia nitrogen* (see Tables V and XIII).

The average amount of ammonia nitrogen present in 100 g. fresh liver is 8·0 mg., the total variation is ± 4·6 mg. or ± 57·9 per cent.; the average for the muscle is rather higher, 10·8 mg. with a total variation of ± 5·5 mg. or of ± 51·2 per cent. The minimum values occur in the same dog but the maximum values do not coincide; in 57 per cent. of the animals the values are correspondingly high or low in both liver and muscle.

With regard to the relation of ammonia to the total soluble nitrogen, the value is correspondingly high or low in 56 per cent. of the experiments in the case of the liver and in 66 per cent. in that of the muscle.

Percentage distribution of the amino, amide and ammonia nitrogen in the total soluble nitrogen.

An examination of the average initial values for the whole series shows clearly that the percentage of the different forms of non-protein nitrogen in the total soluble nitrogen differs quite definitely in the liver and muscle; the percentage of amino acid and of amide nitrogen is considerably greater in the liver than in the muscle, and on the other hand the percentage of undetermined nitrogen in the latter is higher than in the liver; there is therefore a characteristic distribution of the constituents of the non-protein nitrogen for each tissue.

TABLE VIII.

Average values for the different groups of experiments expressed as a percentage of total soluble or non-protein nitrogen.

	Liver						Muscle					
	Percentage of Amino acid N		Percentage of Amide N		Percentage of Ammonia N		Percentage of Amino acid N		Percentage of Amide N		Percentage of Ammonia N	
Experiments	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Early ammonia	23·4	22·8	13·3	15·2	3·2	3·7	16·5	15·5	9·3	9·6	3·2	3·4
Last ammonia	24·6	21·8	14·4	16·6	1·7	8·9	22·7	21·7	11·6	12·7	3·4	4·1
Sodium chloride	28·6	27·3	13·7	13·4	2·7	2·8	21·0	21·3	9·4	9·2	2·7	2·9
Glycocoll	24·1	36·3	15·5	14·8	2·8	4·3	23·2	24·2	12·6	12·7	2·5	2·4
Urea	25·5	20·5	16·2	35·9	2·6	4·0	21·5	19·0	11·4	21·1	2·5	2·7
Average value for whole series	26·1	29·9	14·1	17·2	2·7	4·9	20·7	20·7	10·1	12·0	2·8	3·2

In this connection attention may be directed to the influence exerted by the presence of acid or alkali on the characteristic nitrogen distribution of the autolysing liver in vitro [Bostock, 1912, 2]. The addition of alkali causes the production of a higher percentage of ammonia and of a lower percentage of amide and amino acid nitrogen, whereas the addition of acid brings about a diminution in the percentage of ammonia and an increase in the percentage of amide and amino acid nitrogen. Whether the preponderance of bases in muscle tissue is in any way responsible for the characteristic distribution of nitrogen in this tissue would be difficult to determine, but it approximates more closely to the distribution in autolysing liver in presence of alkali than to that of normal autolysing liver, while it differs from autolysing liver in presence of acid more than from normal autolysing liver.

Effect of Injection of Various Substances on Tissue Composition.

Having studied the variations in the constituents of the nitrogenous material of the tissues which occur in the liver and muscle of normal dogs we may pass to a consideration of the effects produced by the introduction of physiological salt solution and of different nitrogen-containing solutions. The same aspects of tissue analysis will be dealt with as in the section on physiological variation and in a similar order with the exception of the total nitrogen, which has not been treated by itself but only in relation to its constituents.

Table IX contains data regarding the amount of the particular substances and the volume of fluid injected together with the amount of nitrogen introduced per kilo. body weight in the different experiments. It will be seen that in all the earlier work, including all the experiments with sodium chloride and the first three with glycocoll and the ammonia experiments except the last three, dilute solutions practically isotonic with physiological salt solution were used because it was thought that this would interfere less with the normal water content of the tissues. In the later experiments much more concentrated solutions were used and with more striking results as both the glycocoll and ammonia experiments show. It should be mentioned that in the last ammonia experiment 90 cc. in all of a 10 per cent. solution of glucose were introduced alternately with the solution of ammonia. With regard to the urine secreted during the operation, as a rule in the ammonia experiments little or no urine was found in the bladder on the death of the animal, so that the amount of ammonia excreted by this

TABLE IX.

Giving all the experiments and data as to weight of the animal and amount of nitrogen per kilo. body weight contained in the injected fluid, etc., etc.

Experiments		Kilos. body weight	g. of substance injected	g. N in amount injected	g. N per kilo. body wt.	Duration of injection in minutes	Strength of solution	cc. fluid injected	Diet previous to experiment	Remarks
Early ammonia	I	21	7	1·673	0·079	5	isotonic	—	—	—
,,	II	7	2·25	0·537	0·076	25	,,	—	milk and tapioca	died during injection
,,	III	20	4·28	1·022	0·051	33	,,	—	meat	—
,,	IV	6·7	2·98	0·712	0·106	30	,,	—	milk and tapioca	died during injection
,,	V	7	4·79	1·114	—	120	,,	—	milk, lard, tapioca	killed 10 min. after injection
,,	VII	11	2·66	0·635	—	48	,,	—	meat	,, ,,
,, (goat)	VIII	—	—	—	—	—	—	—	—	—
,,	*	—	1·97	0·47	—	47	isotonic	197	tapioca, lard	died during injection
,,	A	—	5·4	1·323	—	60	,,	545	—	—
,,	B	15	6	1·47	0·098	75	,,	600	tapioca, lard	—
,,	C	6·65	2	0·49	0·073	30	,,	20·2	meat, dog biscuit	died during injection
,,	D	7·2	3·7	0·906	0·125	50	,,	378	,, ,,	,, ,,
,,	E	8·3	5	1·225	0·147	75	,,	500	,, ,,	—
,,	F	6·6	3·24	0·793	0·120	80	,,	—	,, ,,	—
,,	G	9·7	4·02	0·929	0·095	100	,,	402	,, ,,	—
Last ammonia	I	8·3	5·05	0·901	0·106	72	5 %	101	porridge and milk	died during injection
,,	II	6	4·15	0·74	0·123	63	5 %	83	,, ,,	,, ,,
,,	III	7·2	7·15	2·085	0·28	56	5 %	143	,, ,,	,, ,,
Sodium chloride	I	11·25	—	—	—	80	0·9 % NaCl	710	,, ,,	—
,,	II	—	—	—	—	—	,,	—	,, ,,	—
,,	III	7·65	—	—	—	40	,,	1000	,, ,,	—
,,	IV	12·7	—	—	—	—	—	—	,, ,,	died before injection
,,	V	4·73	—	—	—	30	0·9 % NaCl	550	,, ,,	—
Glycocoll	I	6·22	8	1·49	0·24	60	1·6 %	500	,, ,,	—
,,	II	6·62	12·8	2·39	0·36	90	,,	800	,, ,,	—
,,	III	23·5	16	2·98	0·12	46	,,	1000	,, ,,	—
,,	IV	31·9	30	5·6	0·17	50	6 %	500	,, ,,	—
,,	V	13·2	15	2·8	0·21	31	6 %	250	,, ,,	—
,,	VI	6·5	13·3	2·48	0·38	49	10 %	133	,, ,,	—
Urea	I	8·9	12·55	5·85	0·65	50	5 %	251	,, ,,	—
,,	II	6·9	8·05	3·75	0·52	75	5 %	161	,, ,,	—

channel must have been very small. In the glycocoll experiments on the other hand the bladder was quite frequently full at the close of the experiment and the amount of amino acid excreted can be seen in Table X; in no case does it amount to more than 20 per cent. of the total amount injected. A considerable amount of amide nitrogen was excreted in the second urea experiment; unfortunately the urine in the case of the first urea experiment was lost.

TABLE X.

Giving the amount of total nitrogen, amino acid, amide and ammonia nitrogen in mg. excreted in the urine secreted during the period of the injection.

Experiments		Total Nitrogen	Amino acid Nitrogen	Amide Nitrogen	NH_3 Nitrogen
Glycocoll	I	602	**304** = amino acid + NH_3 nitrogen		
,,	II	1062	312·6	—	51·8
,,	III	267	14·3	—	11·5
,,	IV	611	355	—	28·5
,,	V	—	456·9	—	23·2
,,	VI	215	158·5	—	6·1
Urea	II	730	—	670	28

If one wishes to produce the maximum fixation by the tissues of the various nitrogen-containing substances introduced into the blood stream, then undoubtedly this is most readily brought about by having a high concentration of the substance in the blood. This requisite concentration is best obtained by the rapid introduction of a concentrated solution of the substance under examination. This is well exemplified in the case of the experiments with urea (see Table V) when the amount injected corresponded to 0·6 and 0·5 g. nitrogen respectively per kilo. body weight.

TABLE XI.

Giving average percentage of dried tissue in 100 *g. moist tissue in the different groups of experiments.*

Experiments	Liver		Muscle	
	Before	After	Before	After
Early ammonia ...	27·26	25·58	25·71	25·02
Last ammonia ...	24·3	23·1	22·7	23·0
Sodium chloride ...	27·39	25·98	25·56	25·95
Glycocoll ...	26·18	23·91	24·40	24·27
Urea	26·0	26·4	23·9	24·06
Average for whole series	26·64	25·05	24·89	24·72

The effect of intravenous injection on the percentage of dried tissue in fresh liver and muscle (see Tables I and XI). The percentage of dried tissue in the fresh liver after injection is 25·05 and is rather smaller than the initial value, the total variation is ± 3·36 or ± 13·4 per cent., a rather wider range than was found before the injection. In 77 per cent. of the experiments the percentage is smaller after injection than in the initial values, showing that in these cases the injection has increased the water content of the tissue. The average for the muscle is 24·7 per cent. and is practically the same as the initial value, the total variation is ± 2·9 or ± 11·7 per cent. In half the experiments the initial value is greater than the value after injection, in the remaining half this condition is reversed. A study of the individual experiments or groups of experiments shows that the water content of the muscle varies as the result of the injection much less than that of the liver. It is quite evident, however, in both cases that the changes in the water content of these tissues offer a totally inadequate explanation for all the variations in the different forms of nitrogen which have been observed. This is particularly the case in those experiments where definite changes were produced in the various nitrogenous constituents of the total soluble nitrogen by the injection of urea, glycocoll and ammonia.

TABLE XII.

(*a*) *Giving total insoluble or protein nitrogen in g. per* 100 *g. fresh tissue, calculated as the average value for each group of experiments.*

Experiments	Liver		Muscle	
	Before	After	Before	After
8 early ammonia ...	2·636	2·700	3·120	2·935
Last ammonia ...	2·719	2·716	2·761	2·837
Sodium chloride ...	2·729	2·765	3·049	3·036
Glycocoll	2·885	2·748	3·046	3·263
Urea	3·032	3·088	2·909	2·921
Mean for whole series	2·772	2·701	3·018	3·013

(*b*) *Giving total insoluble or protein nitrogen in g. per* 100 *g. dried tissue, calculated as the average value for each group of experiments.*

Experiments	Liver		Muscle	
	Before	After	Before	After
Early ammonia ...	9·669	10·55	12·13	11·73
Last ammonia ...	11·19	11·75	12·16	12·33
Sodium chloride ...	9·965	10·64	11·93	11·70
Glycocoll	11·01	11·49	12·48	12·57
Urea	11·66	11·69	12·17	12·14
Mean for whole series	10·4	10·8	12·0	12·1

The effect of intravenous injection on the protein nitrogen (see Tables IV and XII). A consideration of the amount of nitrogen injected in the form of ammonia, amide or of amino acid shows how useless it would be to expect to find signs of its influence on the protein nitrogen; the maximum amount injected was 0·065 g. per 100 g. of tissue in the case of the first urea dog.

If the assumption be made that the injected nitrogen is converted into protein and that it is fairly evenly distributed throughout the body, as the protein nitrogen content of the liver under normal conditions is about 2·70 per cent. this would only give a possible increase to 2·76 per cent., a change small enough to be within the limits of experimental error. It will be seen from Table XII that the average value for the whole series in the case of both liver and muscle whether calculated on the fresh or the dried tissue is practically identical before and after injection, with the exception of a slight diminution in the fresh liver compared with the initial value. In 57 per cent. of the individual experiments however, the value after injection is greater than the initial value in the liver while in the remaining percentage the reverse is true; the range of variation is ± 21·3 per cent. The initial value in the muscle is slightly less than the subsequent value in 52 per cent. of the cases and the range of the variation is ± 16·7 per cent.

In 90 per cent. of the experiments a high or low initial value in the liver is associated with a correspondingly high or low value after injection, and this holds good in 84 per cent. of the experiments in the case of the muscle.

The effect of injection on the total soluble nitrogen (see Tables V, XIII and XIV). The results of the injection of the various solutions can best be studied in Table XIII where the average values for each different group are gathered together, for, as might have been expected from the study of the physiological variation in the normal tissues, individual variations after injection are very considerable; the general trend of the experiments becomes quite clear when the average of the group of similar experiments is examined. Starting with a consideration of the changes effected in the soluble nitrogen, it will be seen that in the liver there is an increase in every group, not excluding the sodium chloride group, but the only significant increase is in the urea and glycocoll groups, where it amounts to 12 and 10 per cent. respectively calculated as a percentage of the initial value. In the individual experiments there are exceptions to the general rule about the relation of the initial to the subsequent values, 23 per cent. of these show a diminished value after injection. Attention must be directed to the glycocoll experiments IV, V and

TABLE XIII.

Giving the average values for the different groups of experiments. Figures represent mg. N. in 100 *g.* moist *tissue.*

	Liver								Muscle							
	Total soluble N		Amino acid N		Amide N		Ammonia N		Total soluble N		Amino acid N		Amide N		Ammonia N	
Experiments	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Early ammonia	274	280	64·3	64·0	36·7	42·6	8·8	10·5	376	369	62·3	57·4	35·2	35·7	12·1	12·6
Last ammonia	303	308	74·6	66·1	43·8	51·3	5·2	**27·6**	386	394	87·8	85·8	45	50·4	13·2	**16·4**
Sodium chloride	306	314	87·6	85·8	42	42·2	8·4	8·9	368	365	77·5	77·8	34·7	33·6	10·3	10·7
Glycocoll	326	360	78·6	**130·9**	50·7	53·5	9·2	15·6	380	396	88·2	**96**	47·9	50·6	9·7	9·9
Urea	312	352	79·6	72·4	50·7	**126·6**	8·3	14·4	401	455	86·5	86·9	45·8	**96·0**	12·1	12·6
Average value for the whole series	292	311	76·3	93·1	41·2	53·6	8·0	15·5	379	383	78·7	79·3	38·6	46·3	10·8	12·3

TABLE XIV.

Giving the average values for the different groups of experiments. Figures represent g. N. in 1000 *g.* dried *tissue.*

	Liver								Muscle							
	Total soluble N		Amino acid N		Amide N		Ammonia N		Total soluble N		Amino acid N		Amide N		Ammonia N	
Experiments	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Early ammonia	10·0	10·9	2·35	2·50	1·35	1·67	0·323	0·42	14·6	14·7	2·42	2·38	1·36	1·43	0·469	0·505
Last ammonia	13·6	16·4	3·30	2·86	1·80	2·21	0·220	1·18	17·0	17·1	3·84	3·72	1·98	2·18	0·58	0·715
Sodium chloride	11·2	12·2	3·03	2·95	1·54	1·62	0·306	0·342	14·4	14·1	3·04	3·00	1·36	1·29	0·400	0·41
Glycocoll	12·4	15·0	3·00	5·80	1·94	2·24	0·352	0·650	15·6	16·3	3·60	3·96	1·95	2·06	0·396	0·405
Urea	12·1	13·3	3·03	2·74	1·93	4·75	0·317	0·545	16·7	18·9	3·61	3·60	1·91	4·00	0·425	0·535
Average for whole series	10·9	12·4	2·86	3·65	1·52	2·10	0·300	0·618	15·2	15·4	3·16	3·20	15·5	18·7	0·440	0·495

VI, where after the injection two separate portions of liver were analysed, there is a considerable difference in the figures obtained for the different portions, but this is not surprising in the light of van Slyke's discovery, already referred to, that the amino acid content of different lobes may vary in a very marked degree. The soluble nitrogen of the muscle only shows a definite increase, amounting to 13 per cent. in the urea group; in the sodium chloride and early ammonia groups it is slightly less than the initial value, and this condition is present in 40 per cent. of the individual experiments. It may be observed that when the values are calculated on the dried muscle instead of on the fresh tissue it is only in the sodium chloride group that the initial is greater than the subsequent value, and even here the difference is really negligible. The total variation in the muscle after injection is ± 19 per cent. and is actually smaller than the variation in the initial values.

The effect of intravenous injection on the ratios of non-protein and protein nitrogen respectively to the total nitrogen (see Tables VI and XV). The average value for the ratio of the non-protein nitrogen in the liver after injection is rather higher than the initial value and amounts to 10·3 per cent., the total variation is ± 3·47 or ± 33·6 per cent. In 27 per cent. of the experiments, however, the initial value is the greater of the two and in 4 per cent. it is unaltered, only in the urea and glycocoll groups is the average value for the group definitely increased by the injection.

The average for the muscle is 11·2 per cent. with a total variation of ± 2·32 or of ± 20·7 per cent. although the average after injection is slightly greater than that of the initial value; in 27 per cent. of the experiments this condition is reversed and in 5 per cent. it is unaltered, only in the urea group is there a definite increase due to the injection.

The total variations of the ratios of the protein nitrogen to total nitrogen are of course the same as those of the ratio of the non-protein to total nitrogen, but expressed as percentage variations they only amount to ± 3·8 per cent. for the liver and ± 2·2 per cent. for the muscle and are practically the same as those of the initial values.

Effect of the injections on the amino acid nitrogen (see Tables V and XIII). Turning now to the amino acid nitrogen, it appears that only in the amino acid group is the value for the liver increased after injection, in all the other groups and in 52 per cent. of the individual experiments the initial value is slightly the greater of the two. The increase in the case of the glycocoll group is very marked and amounts to 66 per cent. In the muscle the effect of the injection of glycocoll is much less pronounced and there is a

comparatively slight increase of 8·8 per cent.; in 33 per cent. of the individual cases the initial value is greater than the subsequent value.

Effect of the injections on the amide nitrogen (see Tables V and XIII). The amide figures are particularly interesting; in the sodium chloride group for the liver they are practically identical before and after injection but in all the other groups there is a definite increase, which amounts to 5 per cent. after the injection of glycocoll, 16 per cent. and 17 per cent. after the injection of ammonia in the first and second group respectively, and 149

TABLE XV.

(*a*) *Giving the average figure for the different groups of experiments of the total soluble or non-protein nitrogen as a percentage of the total nitrogen.*

Experiments	Liver		Muscle	
	Before	After	Before	After
Early ammonia ...	9·4	9·8	10·45	10·93
Last ammonia ...	10·02	10·18	12·26	12·19
Sodium chloride ...	10·2	10·19	10·85	10·75
Glycocoll	10·14	11·61	11·10	10·84
Urea	9·32	10·2	12·11	13·47
Mean for whole series	9·5	10·3	11·1	11·2

(*b*) *Giving the average figure for the different groups of experiments of the protein nitrogen as a percentage of the total nitrogen.*

Experiments	Liver		Muscle	
	Before	After	Before	After
Early ammonia ...	90·5	90·0	89·5	89
Last ammonia ...	89·9	89·8	87·7	87·8
Sodium chloride ...	89·7	89·8	89·1	89·2
Glycocoll	89·9	88·2	88·9	89
Urea	91·6	88·8	87·8	86·5
Mean for whole series	90·5	89·7	88·9	88·8

per cent. in the case of the injection of urea itself. The heaping up of urea is very striking and is obviously determined by the concentration in the blood, whereas in the case of the other groups the increase of amide nitrogen is presumably due to the conversion of some of the injected ammonia or glycocoll. In 11 per cent. of the individual experiments the amide nitrogen is diminished instead of being increased after injection. Fiske and Karsner [1913] have already been mentioned as having perfused the livers of cats and rabbits with defibrinated blood to which additions of ammonium carbonate or of glycocoll were made. They found a definite increase of urea in the blood

from the ammonium salt but none in the case of glycocoll, but as has already been pointed out the conditions of the experiment were not the most favourable for urea formation.

Jansen [1915] perfused the livers of cats and dogs with ammonium carbonate and various amino acids and found an increase of urea in the blood.

In some experiments [Bostock, 1912, 2] which were carried out on the formation of amide nitrogen from glycocoll by the liver pulp in vitro no deaminisation of the glycocoll could be observed; the evidence on the whole, however, was in favour of a small amount of urea formation, for both actual and percentage amounts of amide nitrogen were increased, but the percentage was smaller than the actual increase.

The effect of injection on the ammonia (see Tables V and XIII). The total amount of ammonia in fresh tissue is very small only amounting to 2 or 3 per cent. of the total soluble nitrogen; this amount is practically unchanged after the injection of physiological salt solution but all the other groups show a definite actual and percentage increase as a result of injection. There is an increase of 19 per cent. in the early ammonia experiments, 69 per cent. in the glycocoll group, 73 per cent. in the urea and 430 per cent. in the later ammonia group. The amount of ammonia that can be injected is strictly limited by its toxicity and it would appear that some animals are more susceptible than others; the rate of injection, as well as the amount, exercises an influence on the heaping up in the tissues, the best result being obtained when the organism is flooded with the salt in question. The operation of one or other of these factors probably accounts for the greater accumulation of ammonia found in the later ammonia experiments. In 8 per cent. of the whole series of experiments the ammonia is diminished, in 8 per cent. it is unaltered and in the remaining 84 per cent. it is increased after injection.

It is very interesting to note that there has been a deaminisation of glycocoll, for Lang [1904] claimed that the same thing occurred in vitro, and some earlier experiments of my own with liver pulp seemed to indicate a slight deaminisation; the result of later work however failed to confirm this and showed the necessity of determining the soluble nitrogen as well as the ammonia in all autolysis experiments. An increase of ammonia is not necessarily derived from the amino acid added but may be due to a more rapid autolysis in the case of a particular digest or even to the presence of putrefactive organisms. In every case the percentage relationship of the ammonia to the soluble nitrogen is the important factor, and this relationship

could not be determined in Lang's or in my own early work because the sole estimations were those of ammonia.

The increase of ammonia after the injection of urea is an indication of a certain amount of deaminisation. Wakeman and Dakin [1911] perfused dogs' livers with urea, but under the conditions of their experiments they were unable to detect any increase of ammonia in the blood at the expense of the urea. Janney [1911] investigated the question of whether the conversion of ammonia into urea was a reversible reaction, and after reducing the excretion of ammonia to a minimum by giving large doses of sodium citrate he administered urea but it was not followed by any increase of ammonia in the urine. Lichtwitz [1912] on the other hand criticised Janney's work adversely. He claimed to have found an increase of ammonia and of amino acid with a retention of nitrogen following on the administration of urea to one dog and to four diabetics, but his data are wholly inadequate to establish his conclusions. Marshall and Davies [1914] in their experiments on the distribution and elimination of urea found no evidence of the conversion of urea into any other body but this conclusion is apparently based on indirect evidence as there is no mention of any ammonia estimations.

The changes in the ammonia content of the muscle are much less marked than in the liver; there is an increase in the majority of individual cases, but the amount is unaltered in 18 per cent. and diminished in 9 per cent. of the experiments. In the latest ammonia experiments there is an increase of 24 per cent. but in the other groups the changes are insignificant.

When the values for the non-protein nitrogen and its constituents are calculated on the dried instead of on the fresh tissue they do not materially affect the results, as can be seen from the Tables XIII and XIV, the essential features remain the same and in some cases are even emphasised, as in the urea and glycocoll groups.

When the amino, amide and ammonia nitrogen are calculated as percentages of the non-protein nitrogen it will be seen from Table VIII that the percentage relationships reflect the changes found in the absolute values. Owing to the considerable increase in the value of the non-protein nitrogen itself after the injection of urea and glycocoll, however, the percentage increase in the amide and the amino acid nitrogen in these two groups is less marked than the absolute increase.

As Table XIII is the most important of the whole series it will be well to sum up the results it contains. The sodium chloride group is the only one in which all the values non-protein, amino, amide and ammonia nitrogen,

both total and percentage, remain practically unchanged. Both the ammonia groups show a total and percentage increase in the ammonia and amide nitrogen of the liver after injection, which can only be interpreted as due to the fixation of ammonia and the conversion of some of it into urea. When the organism is flooded with ammonia, to use van Slyke's phrase, it desaturates itself by working it up into the normal product, but whether this is the only way in which desaturation takes place, the data available do not indicate. Another point of interest is that there is no indication of an amination of fatty acids occurring; possibly suitable conditions are not present, but at any rate we are justified in assuming that if amination occurs it is a much more specialised process than the synthesis of urea. Turning to the muscle in the last ammonia group the same changes are observed in a less marked degree than in the liver, but they are scarcely perceptible in the group of earlier experiments. Undoubtedly there is a fixation of ammonia by the muscle and it seems probable that the muscle possesses the power of converting this into urea without the intervention of the liver.

The glycocoll group shows a very marked fixation of amino acid by the liver and quite a marked increase in the ammonia due to a deaminisation of the glycocoll; there is also a definite but less well marked increase in the amide nitrogen due to a conversion of some of the ammonia into urea. An increase of urea in the blood after the intravenous injection or absorption of large quantities of amino acid has been abundantly shown by van Slyke and by Folin, but I am not aware of the same changes having been demonstrated in the liver, although Folin and his pupils have shown a very marked increase in the urea of the muscle under these conditions. The fixation of amino acid by the muscle is much smaller than by the liver; van Slyke has shown that it does not reach its saturation point as soon as the liver. It is to be noted that although the ammonia has not increased there is a definite increase in the amide nitrogen.

The urea group reveals changes in the ammonia and amide nitrogen, the latter is enormously increased and the former shows a very definite increase, and there can be no doubt of the deaminisation of some of the urea. The muscle also exhibits a high degree of urea fixation and a slight increase in the ammonia nitrogen.

Conclusions.

Variation is the rule in those aspects of tissue analysis which form the subject of the present communication, there is not a single instance of a constant value or relation although the range of variation is much greater in some relations than in others.

The intravenous injection of ammonia, urea and glycocoll, respectively, produces an accumulation of these bodies as such in the liver and to a less extent in the muscle. There is no evidence of the synthesis of any of these substances into protein but there is abundant proof of their further metabolism particularly in the liver; ammonia is converted into amide nitrogen in both liver and muscle, urea is deaminised in the liver and muscle while glycocoll is deaminised in the liver and is partially converted into amide nitrogen in both liver and muscle.

The expenses of this investigation were in part defrayed by a grant from the Royal Society and in part by a grant from the Carnegie Trust.

It is a great pleasure to express my indebtedness to Dr E. P. Cathcart for his help and criticism throughout.

REFERENCES.

Abderhalden (1912, 1). *Zeitsch. physiol. Chem.*, **77**, 22.
—— (1912, 2). *Zeitsch. physiol. Chem.*, **78**, 1.
—— (1913). *Zeitsch. physiol. Chem.*, **83**, 444.
Abderhalden and Hirsch (1912, 1). *Zeitsch. physiol. Chem.*, **78**, 292.
—— (1912, 2). *Zeitsch. physiol. Chem.*, **80**, 136.
—— (1912, 3). *Zeitsch. physiol. Chem.*, **81**, 323.
—— (1912, 4). *Zeitsch. physiol. Chem.*, **82**, 1.
—— (1913). *Zeitsch. physiol. Chem.*, **84**, 189.
Abderhalden and Lampe (1912, 1). *Zeitsch. physiol. Chem.*, **80**, 160.
—— (1912, 2). *Zeitsch. physiol. Chem.*, **82**, 21.
—— (1913, 1). *Zeitsch. physiol. Chem.*, **83**, 409.
—— (1913, 2). *Zeitsch. physiol. Chem.*, **84**, 218.
Bayliss (1915). *Principles of General Physiology.*
Bostock (1912, 1). *Biochem. J.*, **6**, 48.
—— (1912, 2). *Biochem. J.*, **6**, 388.
Buglia (1912, 1). *Zeitsch. Biol.*, **57**, 365.
—— (1912, 2). *Zeitsch. Biol.*, **58**, 162.
Buglia and Constantino (1913). *Zeitsch. physiol. Chem.*, **84**, 243.
Diesselhorst (1911). *Pflüger's Arch.*, **140**, 256.
Embden and Schmitz (1912). *Biochem. Zeitsch.*, **38**, 393.
Emmet and Carroll (1911). *Maly's Jahresbericht*, 552.
Fiske and Karsner (1913). *J. Biol. Chem.*, **16**, 399.
Fiske and Sumner (1914). *J. Biol. Chem.*, **18**, 285.
Folin and Denis (1912, 1). *J. Biol. Chem.*, **11**, 87.
—— (1912, 2). *J. Biol. Chem.*, **11**, 161.

Folin and Denis (1912, 3). *J. Biol. Chem.*, **12**, 141.
—— (1912, 4). *J. Biol. Chem.*, **12**, 253.
—— (1912, 5). *J. Biol. Chem.*, **12**, 259.
Frank and Schittenhelm (1910). *Zeitsch. physiol. Chem.*, **70**, 98.
—— (1911). *Zeitsch. physiol. Chem.*, **73**, 157.
Gammeltoft (1911). *Zeitsch. physiol. Chem.*, **75**, 57.
Grafe (1912, 1). *Zeitsch. physiol. Chem.*, **78**, 485.
—— (1912, 2). *Zeitsch. physiol. Chem.*, **82**, 347.
—— (1913, 1). *Zeitsch. physiol. Chem.*, **84**, 69.
—— (1913, 2). *Zeitsch. physiol. Chem.*, **86**, 347.
—— (1913, 3). *Zeitsch. physiol. Chem.*, **88**, 389.
—— (1914). *Zeitsch. physiol. Chem.*, **90**, 75.
Grafe and Schläpfer (1912). *Zeitsch. physiol. Chem.*, **77**, 1.
Grafe and Turban (1913). *Zeitsch. physiol. Chem.*, **83**, 25.
Grafe and Wintz (1913). *Zeitsch. physiol. Chem.*, **86**, 283.
Grund (1910). *Zeitsch. Biol.*, **54**, 173.
György and Zunz (1915). *J. Biol. Chem.* **21**, 511.
Henderson, Lawrence (1911). *J. Biol. Chem.*, **9**, 403.
Henriques and Anderson (1913). *Zeitsch. physiol. Chem.*, **88**, 357.
—— (1914, 1). *Zeitsch. physiol. Chem.*, **92**, 21.
—— (1914, 2). *Zeitsch. physiol. Chem.*, **92**, 194.
Janney (1911). *Zeitsch. physiol. Chem.*, **76**, 99.
Jansen (1915). *J. Biol. Chem.*, **21**, 557.
Kellner, Eisenkolbe, Flebbe and Neumann (1910). *Land. Vers. Stat.*, **72**, 437.
Knoop and Kertess (1911). *Zeitsch physiol. Chem.*, **71**, 252.
Lang (1904). *Beiträge*, **5**, 321.
Lichtwitz (1912). *Zeitsch. physiol. Chem.*, **77**, 402.
McCollum (1911). *Amer. J. Physiol.*, **29**, 215.
McLean and Selling (1914). *J. Biol. Chem.*, **19**, 31.
Marshall and Davies (1914). *J. Biol. Chem.*, **18**, 53.
Matthews and Nelson (1914). *J. Biol. Chem.*, **19**, 229.
Morgan, Beger and Westhausser (1910). *Land. Vers. Stat.*, **73**, 285.
—— (1911). *Land. Vers. Stat.*, **75**, 265.
Oehme (1914). *Zeitsch. physiol. Chem.*, **89**, 312.
Osborne and Mendel (1912, 1). *Zeitsch. physiol. Chem.*, **80**, 307.
—— (1912, 2). *J. Biol. Chem.*, **12**, 473.
Pfeiffer and Kriske (1911). *Land. Vers. Stat.*, **74**, 409.
Schryver (1906). *Biochem. J.*, **1**, 123.
Shermann and Gettler (1912). *J. Biol. Chem.*, **11**, 323.
Slyke, van (1913). *J. Biol. Chem.*, **16**, 187.
Slyke, van, and Meyer (1912). *J. Biol. Chem.*, **12**, 399.
—— (1913, 1). *J. Biol. Chem.*, **16**, 197.
—— (1913, 2). *J. Biol. Chem.*, **16**, 213.
—— (1913, 3). *J. Biol. Chem.*, **16**, 231.
Taylor and Ringer (1913). *J. Biol. Chem.*, **14**, 407.
Underhill and Goldschmidt (1913, 1). *J. Biol. Chem.*, **15**, 327.
—— (1913, 2). *J. Biol. Chem.*, **15**, 337.
—— (1913, 3). *J. Biol. Chem.*, **15**, 341.
Wakeman and Dakin (1911). *J. Biol. Chem.*, **9**, 327.

XXI. THE INFLUENCE OF THE NATURE OF THE DIET ON THE RETENTION OF PROTEIN.

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(*Received April 21st, 1916.*)

The question of the capacity of the organism to store protein has always been one of considerable interest and although it is highly probable that such a storing is a normal process the critical conditions for its occurrence have not yet been fully elucidated, largely owing to the fact that the amount of information available from nitrogen equilibrium studies is small and its value difficult to assess. Still by the use of the method of superimposition results of importance can be obtained without recourse to such favouring conditions of storage as muscle work, growth or inanition, all of which conditions introduce secondary factors which render the interpretation of the data more difficult.

In the course of another investigation Tsuji [1915] found that the degree of retention of superimposed protein varied (1) with the nature of the standard diet, and (2) with the nature of the protein superimposed. As these experiments were, as regards the superimposition, only of one day's duration the present series of experiments was carried out to amplify the data obtained.

The animal employed was an Airedale bitch weighing 17·6 kilos. She was fed daily at 11 a.m. after catheterisation, the urine then obtained being added to the urine collected in the receiver of the metabolic cage.

The analytical methods employed were: total nitrogen, Kjeldahl; urea, Plimmer and Skelton's modification of the urease method; ammonia, Folin. The delimitation of the faeces was carried out by means of charcoal or carmine. The total faeces was collected for each period and a single analysis of the mixed specimens was made.

As regards the standard diets employed three different combinations of protein, fat and carbohydrate were used. They all contained the same amount

of protein and were of the same caloric value but varied markedly in their content of fat and carbohydrate. They were as follows:

I. Carbohydrate-rich, fat-poor diet.

Scott's oatflour	= 120 g.		
Dried skimmed milk	= 43 g.		
Margarine	= 34 g.	Nitrogen	7·2 g.
Caseinogen	= 19 g.	Carbohydrate	194 g.
Tapioca	= 93 g.	Fat	34 g.
Sodium chloride	= 2 g.	Caloric intake	1296

II. Intermediate diet.

Scott's oatflour	= 60 g.		
Dried skimmed milk	= 62 g.		
Margarine	= 77 g.	Nitrogen	7·2 g.
Caseinogen	= 19 g.	Carbohydrate	117 g.
Tapioca	= 47 g.	Fat	68 g.
Sodium chloride	= 2 g.	Caloric intake	1296

III. Fat-rich, carbohydrate-poor diet.

Scott's oatflour	= 30 g.		
Dried skimmed milk	= 40 g.		
Margarine	= 120 g.	Nitrogen	7·2 g.
Caseinogen	= 34 g.	Carbohydrate	42 g.
		Fat	101 g.
Sodium chloride	= 2 g.	Caloric intake	1296

The standard diet was continued for a pre-period of five to eight days until the nitrogen output was approximately constant, there was then added to the diet a definite amount of protein material, and this was continued for eight days when the diet again reverted to the original standard for a post-period of six or eight days. It was thought that in this way some definite information as regards retention would be obtained.

Caseinogen experiments.

EXPERIMENT I. *Retention of caseinogen nitrogen on the carbohydrate-rich, fat-poor diet.*

In this experiment the animal was fed on the carbohydrate-rich standard diet. After a pre-period of five days 30 g. of caseinogen containing 3·6 g. nitrogen were added daily to the standard diet during eight days. Then the diet was again changed, for a post-period of seven days, to the original standard diet.

It will be noted from the Table I A that although there was a progressive fall in the retention of nitrogen from + 0·4 g. on the first day to + 0·3 g. on the fourth day and even a slight negative balance on the fifth day the average daily retention was + 0·2 g. giving a total retention of 1·44 g. for the whole

period. During the chief period, I B, when caseinogen was being added to the diet there was a well-marked retention amounting to + 1·26 g. daily, with a total retention for the whole period of 10·0 g. nitrogen. As during this period 28·8 g. of nitrogen were superimposed there was therefore a retention

TABLE I.

A. *Pre-period.*

Day of experiment	Total amount of urine in cc.	Nitrogen intake in g.	Nitrogen output in g.				Daily nitrogen balance g.
			Urine			Faeces	
			Ammonia	Urea	Total nitrogen		
I	690	7·2	0·49	4·98	6·05	0·66	+0·49
II	540	7·2	0·48	5·02	6·10	0·66	+0·44
III	670	7·2	0·48	5·19	6·23	0·66	+0·31
IV	620	7·2	0·46	5·12	6·16	0·66	+0·38
V	560	7·2	0·51	5·60	6·72	0·66	−0·18
Average		7·2	0·48	5·18	6·25	0·66	+0·29

B. *Caseinogen period* (3·6 g. nitrogen in the form of caseinogen were added daily).

VI	700	10·8	0·64	7·13	8·36	0·79	+1·65
VII	700	10·8	0·66	7·24	8·56	0·79	+1·45
VIII	580	10·8	0·67	6·63	7·91	0·79	+2·10
IX	570	10·8	0·80	7·89	9·34	0·79	+0·67
X	730	10·8	0·76	7·73	9·07	0·79	+0·94
XI	730	10·8	0·69	7·22	8·49	0·79	+1·52
XII	770	10·8	0·70	7·61	8·98	0·79	+1·03
XIII	840	10·8	0·78	7·88	9·28	0·79	+0·73
Average		10·8	0·71	7·42	8·75	0·79	+1·26

C. *After period.*

XIV	790	7·2	0·51	6·03	7·26	0·66	−0·72
XV	900	7·2	0·49	5·83	6·93	0·66	−0·37
XVI	800	7·2	0·48	5·07	6·18	0·66	+0·38
XVII	800	7·2	0·55	5·75	6·90	0·66	−0·36
XVIII	760	7·2	0·47	5·27	6·34	0·66	+0·20
XIX	760	7·2	0·54	5·91	7·08	0·66	−0·54
XX	840	7·2	0·50	5·85	6·95	0·66	−0·41
Average		7·2	0·51	5·67	6·81	0·66	−0·26

of just over 35 %. In the post-period, I C, there was a quite definite negative balance, the average daily loss being − 0·26 g. or 1·84 g. for the total period. This loss, as it continues over the whole period, cannot in the light of Falta's [1906] careful experiments on the re-excretion of nitrogen be regarded solely as due to re-excretion. Falta found in his experiments with caseinogen that

the re-excretion ceased about the fourth day after the caseinogen was stopped. Moreover Tsuji's experiments demonstrate clearly that there is no prolonged excretion of excess nitrogen after the superimposition of caseinogen. This prolonged secondary loss is very difficult to explain except on the ground that there has been a stimulation of the total protein metabolism due to the increased intake.

EXPERIMENT II. *Retention of caseinogen nitrogen on the intermediate diet.*

TABLE II.

A. *Pre-period.*

Day of experiment	Total amount of urine in cc.	Nitrogen intake in g.	Nitrogen output in g.				Daily nitrogen balance g.
			Urine			Faeces	
			Ammonia	Urea	Total nitrogen		
I	590	7·2	0·42	5·94	6·99	0·56	−0·35
II	560	7·2	0·40	5·33	6·32	0·56	+0·32
III	530	7·2	0·46	5·65	6·74	0·56	−0·10
IV	620	7·2	0·45	5·64	6·76	0·56	−0·12
V	590	7·2	0·47	5·99	7·15	0·56	−0·51
Average		7·2	0·44	5·71	6·79	0·56	−0·15

B. *Caseinogen period* (3·6 g. nitrogen in the form of caseinogen were added daily).

VI	620	10·8	0·68	7·68	9·07	0·65	+1·08
VII	520	10·8	0·72	7·77	9·18	0·65	+0·97
VIII	610	10·8	0·67	7·28	8·69	0·65	+1·46
IX	530	10·8	0·69	7·54	8·85	0·65	+1·30
X	670	10·8	0·69	7·66	9·00	0·65	+1·15
XI	590	10·8	0·78	8·00	9·41	0·65	+0·74
XII	610	10·8	0·73	8·16	9·54	0·65	+0·61
XIII	460	10·8	0·71	7·85	9·23	0·65	+0·92
Average		10·8	0·71	7·74	9·12	0·65	+1·03

C. *After period.*

XIV	560	7·2	0·50	5·34	6·36	0·84	0
XV	580	7·2	0·47	5·80	6·90	0·84	−0·54
XVI	490	7·2	0·48	5·18	6·27	0·84	+0·09
XVII	610	7·2	0·47	5·04	6·07	0·84	+0·29
XVIII	540	7·2	0·43	5·11	6·09	0·84	+0·27
Average		7·2	0·47	5·29	6·34	0·84	+0·02

In this experiment the standard diet employed was the intermediate one and 30 g. of caseinogen were added during the chief period. During the after period there was a certain amount of difficulty as the faeces became semi-diarrhoeic and we may conclude that absorption from the intestine was

imperfect. During the pre-period the nitrogen balance was, with the exception of one day, slightly on the negative side giving a daily average of −0·15 g., *i.e.* 0·76 g. was lost during this period. When caseinogen was added the balance was positive throughout the period giving a daily average of + 1·03 g. the total retention for the period being 8·23 g., *i.e.* 28·6 % of the nitrogen added in the form of caseinogen was retained. In the post-period there was a slight positive balance.

EXPERIMENT III. *Retention of caseinogen nitrogen on the fat-rich, carbohydrate-poor diet.*

TABLE III.

A. *Pre-period.*

Day of experiment	Total amount of urine in cc.	Nitrogen intake in g.	Nitrogen output in g.				Daily nitrogen balance g.
			Urine			Faeces	
			Ammonia	Urea	Total nitrogen		
I	360	7·2	0·45	5·44	6·56	0·73	−0·09
II	330	7·2	0·50	5·30	6·52	0·73	−0·05
III	350	7·2	0·54	5·27	6·48	0·73	−0·01
IV	400	7·2	0·56	5·33	6·54	0·73	−0·07
V	610	7·2	0·58	5·67	6·79	0·73	−0·32
Average		7·2	0·53	5·40	6·58	0·73	−0·11

B. *Caseinogen period* (3·6 g. nitrogen in the form of caseinogen were added daily).

VI	610	10·8	0·81	8·01	9·44	0·67	+0·69
VII	440	10·8	0·84	7·79	9·32	0·67	+0·81
VIII	600	10·8	0·88	8·32	9·97	0·67	+0·16
IX	750	10·8	1·03	8·49	10·21	0·67	−0·08
X	690	10·8	0·88	8·50	10·08	0·67	+0·05
XI	680	10·8	0·90	8·33	9·86	0·67	+0·27
XII	690	10·8	0·89	8·36	9·97	0·67	+0·16
XIII	660	10·8	0·87	8·13	9·74	0·67	+0·39
Average		10·8	0·89	8·24	9·82	0·67	+0·31

C. *After period.*

XIV	852	7·2	0·66	5·14	6·45	0·76	−0·01
XV	620	7·2	0·66	5·35	6·65	0·76	−0·21
XVI	640	7·2	0·63	5·38	6·65	0·76	−0·21
XVII	620	7·2	0·59	5·24	6·43	0·76	+0·01
XVIII	850	7·2	0·58	4·85	6·18	0·76	+0·26
Average		7·2	0·62	5·19	6·47	0·76	−0·03

In this experiment the dog was kept throughout on the fat-rich diet, 30 g. of caseinogen being added daily during the chief period.

During the pre-period, as in the second experiment, there was a slight negative balance which amounted to a daily loss of − 0·11 g. or of 0·54 g. nitrogen in all. In the chief period except for the first day when there was a small negative balance there was evidence of retention of nitrogen. The average balance for the eight days was + 0·31 g. and the total amount retained was 2·45 g. or 8·5 % of the total nitrogen ingested in the form of caseinogen. In the post-period during the first three days the balance was negative and on the two following days positive, giving however an average loss of − 0·03 g. nitrogen per diem.

One fact emerges very clearly from the foregoing experiments namely that the nature of the standard diet employed plays a well-defined part in the degree of retention obtained. In each experiment the same amount of nitrogen in the form of caseinogen was added to the diet but the amount retained varied in each instance. Thus in the case of the carbohydrate-rich diet there was a retention of 10·09 g. corresponding to 35 % of that superimposed; on the intermediate diet the retention amounted to 8·23 g. or 28·6 % of the superimposed amount and on the carbohydrate-poor, fat-rich diet the retention amounted only to 2·45 g., equivalent to 8·5 % of the total addition. The evidence, then, clearly points in favour of the view that carbohydrate plays some preponderant *rôle* in the retention of protein, that when a sufficiency of carbohydrate is not available there is a failure in the synthetic processes. This is borne out by the consideration, for example, of the marasmic condition associated with diabetes, where protein is given in large amount in the diet but without any gain on the part of the patient. It had been hoped that some light would have been obtained on the proportion of carbohydrate which was essential, *i.e.* on the ratio which must exist between carbohydrate and fat in a diet, but, owing to various difficulties, this point could not be fully investigated at present. From the results of Lang [1915] on acidosis it would seem probable however that the highest ratio of fat to carbohydrate which is possible is 1 : 2.

Caseinogen is generally recognised as being a form of protein which is more readily retained than many other forms [Röhmann 1898; Caspari 1900], although this has been denied by some workers [Bloch 1900] for example. As the present experiments had given such a satisfactory result the question as to whether the superimposition of an imperfect protein like gelatin would yield an equally satisfactory result arose.

EXPERIMENT IV. *Retention of gelatin nitrogen on the carbohydrate-rich, fat-poor diet.*

TABLE IV.

A. *Pre-period.*

Day of experiment	Total amount of urine in cc.	Nitrogen intake in g.	Nitrogen output in g.				Daily nitrogen balance g.
			Urine			Faeces	
			Ammonia	Urea	Total nitrogen		
I	800	7·2	0·48	5·07	6·18	0·66	+0·38
II	800	7·2	0·55	5·75	6·90	0·66	−0·36
III	760	7·2	0·47	5·27	6·34	0·66	+0·20
IV	760	7·2	0·54	5·91	7·08	0·66	−0·54
V	840	7·2	0·50	5·85	6·95	0·66	−0·41
Average		7·2	0·51	5·57	6·69	0·66	−0·15

B. *Gelatin period* (4·1 g. nitrogen in the form of gelatin were added daily).

VI	840	11·3	0·60	8·87	10·16	0·78	+0·36
VII	840	11·3	0·64	9·04	10·33	0·78	+0·19
VIII	600	11·3	0·60	7·86	9·09	0·78	+1·43
IX	770	11·3	0·58	8·04	9·30	0·78	+1·22
X	880	11·3	0·63	8·69	10·03	0·78	+0·49
XI	860	11·3	0·60	8·37	9·63	0·78	+0·89
XII	760	11·3	0·64	8·39	9·77	0·78	+0·75
XIII	810	11·3	0·60	7·85	9·07	0·78	+1·45
Average		11·3	0·61	8·39	9·67	0·78	+0·85

C. *Gelatin and meat extract period* (4·1 g. nitrogen in the form of gelatin and 0·5 g. nitrogen in the form of meat extract were added daily).

I	760	11·8	0·59	8·77	10·24	0·96	+0·60
II	1020	11·8	0·68	9·55	11·08	0·96	−0·24
III	780	11·8	0·64	8·37	9·72	0·96	+1·12
Average		11·8	0·64	8·90	10·35	0·96	+0·49

D. *After period.*

I	980	7·2	0·58	6·34	7·50	0·97	−1·27
II	1040	7·2	0·56	5·88	7·02	0·97	−0·79
III	960	7·2	0·53	5·35	6·38	0·97	−0·15
Average		7·2	0·55	5·86	6·97	0·97	−0·74

As in the previous experiments the animal was put on the standard diet, in this case the carbohydrate-rich, fat-poor diet, for a pre-period of five days, then to this diet there was added daily during the chief period 30 g. of gelatin containing 4·1 g. nitrogen.

During the pre-period the balance was very variable, the daily average for the period being − 0·15 g. In the chief period the balance throughout

was positive, the average daily retention amounting to + 0·85 g., the total retention for the whole period was 6·78 g. corresponding to 20·7 % of the nitrogen taken in, *i.e.* the retention in the case of gelatin is definitely lower than in that of caseinogen under exactly the same conditions. In view of Thompson's [1910] statement that the addition of meat extract to a diet brings about a more perfect retention it was thought that the present series of experiments offered a good opportunity of testing this matter. Accordingly the chief period was continued for a further three days during which time 5 g. of meat extract were added to the gelatin and superimposed on the standard diet. As the figures in IV C show this addition produced no increase in the amount retained, indeed the daily average was definitely lowered. Unfortunately, as the animal was tiring of the diet, the post-period could not be continued for the usual length of time. (Unless every precaution is taken to see that the animals, during these tests, maintain their appetites the experiments lose a very large part of their value.) During the post-period, in the present experiment, there was a very well-marked negative balance which may be due in large part to a re-excretion of the retained nitrogen. In this respect the apparent retention is even more unfavourable than the above calculation would suggest.

EXPERIMENT V. *Retention of gelatin nitrogen on a fat-rich, carbohydrate-poor diet.*

TABLE V.

A. *Pre-period.*

Day of experiment	Total amount of urine in cc.	Nitrogen intake in g.	Nitrogen output in g.				Daily nitrogen balance g.
			Urine			Faeces	
			Ammonia	Urea	Total nitrogen		
I	750	7·2	0·60	5·47	6·70	0·53	−0·03
II	680	7·2	0·62	5·40	6·59	0·53	+0·08
III	550	7·2	0·58	4·74	5·89	0·53	+0·78
IV	630	7·2	0·62	5·50	6·79	0·53	−0·12
V	640	7·2	0·63	5·84	7·01	0·53	−0·34
Average		7·2	0·61	5·39	6·60	0·53	+0·07

B. *Gelatin period* (4·1 g. nitrogen in the form of gelatin were added daily).

I	530	11·3	0·69	8·66	10·01	0·81	+0·48
II	580	11·3	0·73	8·81	10·15	0·81	+0·34
III	560	11·3	0·62	8·94	10·21	0·81	+0·28
IV	590	11·3	1·20	8·88	9·99	0·81	+0·50
Average		11·3	0·76	8·82	10·09	0·81	+0·40

In this experiment the standard diet was fat-rich, carbohydrate-poor. The amount of gelatin superimposed was the same as in experiment IV. This experiment is not complete as after the fifth day of the chief period the animal lost its appetite and could not be induced to finish its daily allowance of food. The data recorded are all, however, free from objection.

During the pre-period again the balance was variable but in this instance it was very slightly positive, the daily average retention being + 0·07 g. In the chief period the average daily retention amounted to + 0·4 g. or 1·6 g. for the four days corresponding to 9·76 % of the total nitrogen superimposed, a result slightly more favourable than that found for caseinogen under similar conditions for a period of eight days (8·5 %), but slightly less favourable if compared with the retention of the caseinogen nitrogen for the period of the first four days (10·97 %), which is more directly comparable with the gelatin figures. Here, therefore, as in the case of the caseinogen, although the retention is not quite so marked, the carbohydrate exercises a definite retaining influence on the superimposed nitrogen.

As regards the method by which the carbohydrate acts it is not proposed to deal with the problem in the present communication. It is hoped however to take up the consideration of this question in another paper.

Conclusions.

1. Nitrogen in the form of protein added to a carbohydrate diet is retained in greater amount than when added to a fat diet of equal caloric value.

2. Nitrogen given in the form of caseinogen is more completely retained than when given in the form of gelatin.

3. The addition of meat extract to gelatin does not increase the amount of nitrogen retained.

These experiments were carried out at the suggestion and under the direction of Dr E. P. Cathcart to whom I offer my thanks.

The expenses were defrayed by a grant from the London Hospital Research Fund.

REFERENCES.

Bloch (1900). *Zeitsch. diätet. physik. Ther.*, **3**, 482.
Caspari (1900). *Zeitsch. diätet. physik. Ther.*, **3**, 393.
Falta (1906). *Deutsch. Arch. klin. Med.*, **86**, 517.
Lang (1915). *Biochem. J.*, **9**, 456.
Röhmann (1898). *Berl. klin. Woch.*, **35**, 789.
Thompson (1910). *Brit. Assoc. Report*, 760.
Tsuji (1915). *Biochem. J.*, **9**, 439.

XXII. "PLATE" FORMS OF ULTRAFILTRATION APPARATUS.

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(*Received May 4th, 1916.*)

The use of ultrafilters as accepted aids to investigation leads naturally to more practical forms of ultrafiltration apparatus than that in which observations of their properties were originally made. For particular experiments special designs will always be necessary: nevertheless, as an attempt at sound constructions for general laboratory use, which will doubtless be improved upon in the light of wider experience, I put forward those illustrated below. The larger pattern was briefly described in a previous communication in which details of a method of making suitable ultrafilters were also given [Walpole, 1915].

12″ Apparatus—Pattern A. Diameter of Filter 10″.

Construction of the Ultrafiltration Apparatus proper.

There are two circular metal plates 12″ diameter and $\frac{3}{8}$″ thick called the *front plate* and the *back plate* (Fig. 1). These can be bolted together by nine $\frac{3}{8}$″ bolts and three $\frac{3}{8}$″ studs and wing-nuts arranged symmetrically, the former with their centres round an $11\frac{3}{8}$″ circle, and the latter round an $11\frac{5}{16}$″ circle. Between them is a $\frac{3}{16}$″ filter plate built up of two pieces —a ring 11″ external and 10″ internal diameter, and a 10″ circular disc of exactly the same thickness over all and embossed with a pyramidal pattern on both sides. The ring just clears the bolts all round and has three small indentations cut in it enabling it to fit exactly between the three studs. These support the filter plate before the front plate is applied to it, and

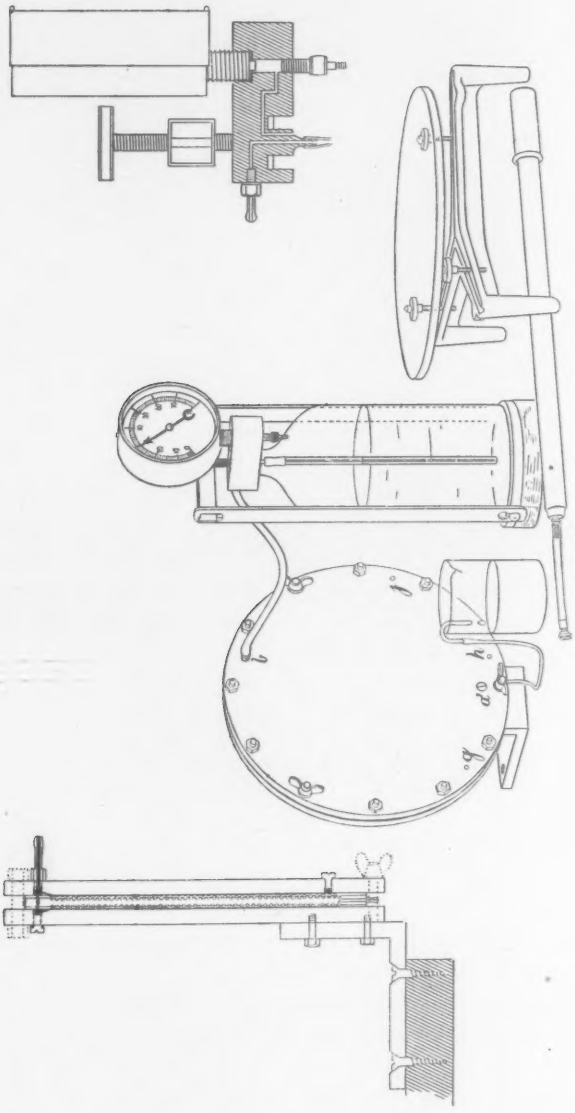

Fig. 1.

ensure that when the films are in position the front plate may be slid on gently with all the bolt holes and the holes *b*, *d*, and *a* (Fig. 2) in perfect register.

In Fig. 2 the two filters themselves are shown in position. They are indicated by the white space between two thin black lines. The metal of the three plates is indicated by cross-hatching and the fluid to be filtered entering the nipple *b* under pressure is represented by dead black. Passing through channels *x* and *y* it spreads all over both filters. The raised "pyramidal pattern" of the filter plate acts as an excellent support for the filters under pressure, and when once the filtrate (not indicated) has passed through the filter it has an unobstructed flow to the edge of the filter plate. Here it can pass through the channel *c'* out of the apparatus. A similar channel *c*, not shown, is at the top of the filter plate and connects to the outside air. The pyramidal pattern filter plate has been used by permission of Messrs S. H. Johnson, the owners of the patent.

It will be noticed that *c*, *c'* are placed in the "median line," as it were, of the apparatus, while *a*, *d*, *b*, in order that the channel they form may not cut into *c*, are placed a little to one side. For convenience of illustration the top half of Fig. 2 is a vertical section through *d*, and the lower half a vertical section through *c'*, the outlet for the filtrate.

The two holes *a*, *b* and another at *a'* are tapped No. 2 B.A. thread. Fitting interchangeably into these three tapped holes are two screwed plugs and a screwed nipple all fitted with small fibre washers.

At *g*, *h*, *j* (Fig. 1) on the front plate, and at similar positions on the back plate, another set of holes is drilled and tapped. These are seldom used, but if, at the end of a filtration, the filter plate is found to adhere to either of the other plates, separation may be immediately and safely effected by screwing brass screws into these holes.

The apparatus may be made of iron varnished and baked, or of aluminium. In the latter case rolled sheet, and not cast metal, must be used, because of the porosity of the latter. Aluminium has many advantages including that of cheapness, for there is a good deal of turning to be done, and aluminium can be cut much more quickly than iron.

To the back plate is attached a bracket, by means of which it may be screwed or clamped to the bench. As a matter of convenience the apparatus is drawn in the position shown. It is used just as easily and just as often in the inverted position which is advantageous for certain operations described later.

Thick-walled rubber tubing of capillary bore is used to lead into the apparatus through the nipple the liquid to be filtered. No wiring is required on such a joint which is perfectly sound up to quite considerable pressures (50–80 lbs. per sq. inch) and may be readily made and unmade. Capillary cycle valve tubing is used to lead off the filtrate through the small nipple at c', and to admit at c air blowing out of c' the last drops of filtrate.

Method of assembling Apparatus for use.

To facilitate description it will be supposed in the first instance that a filtration is to be performed using two filters, one on each side of the filter plate. The circle of plate glass on which the collodion was poured [see Walpole, 1915, pp. 291 et seq.] is 12″ diameter so that the prepared films

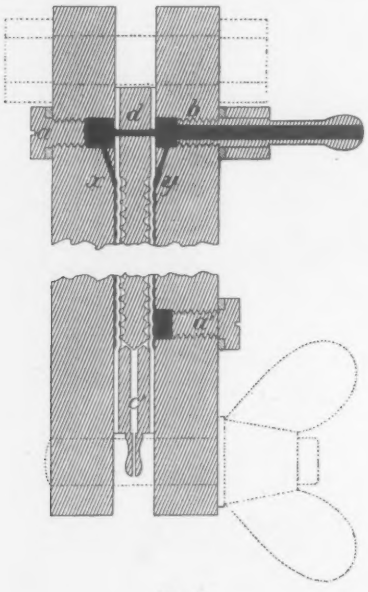

Fig. 2.

lying under water are all this size. One is taken, laid on a clean piece of wood or glass and, using the filter plate as template, is cut round with a knife. The trimmed film 11″ diameter is put back under water and from the cuttings a piece is quickly taken by means of scissors, taking care not to include any part from the extreme edge where oil containing collodion has been

employed to prevent the film curling off the glass during drying. This piece is dried between hard filter paper without using pressure and weighed, and then dried to constant weight at 100° C. and weighed again. The ratio of these two weights gives w the wetness of the film. The value m, milligrams of dry nitro-cotton per sq. cm., is known from the volume and concentration of the collodion taken and the area of the glass plate over which it was poured.

When the two circular films each 11″ diameter are prepared, one is taken and applied carefully to the front face of the *back plate*. As it is wet it is readily manoeuvred into position and then held there by placing the *filter plate* in front of it. As has already been noticed the nipple of the *front plate* is not quite in the median line, so that in order that the continuous channel shown in the top half of Fig. 2 may be formed, care must be taken not to put the *filter plate* in back to front. The second film, thoroughly wet, is now applied to the back face of the *front plate*, the *front plate* slid on to the studs, and the whole apparatus tightened up by screwing up the wing-nuts and bolts seriatim, going round the apparatus several times. Wet films "give" considerably under pressure and the tightening up of the apparatus takes some time. The nipple on the *front plate* and the screw opposite it on the back plate are now removed and a $\frac{3}{32}$″ punch passed through so that two neat circular holes are made in the films. The screw and nipple are replaced, the rubber supply tube connected to the nipple, and filtration may be commenced forthwith. The liquid under pressure set up by the cycle pump and indicated by the gauge is forced through the passage shown in Fig. 2 over both films and the combined filtrates run away through c'. By connecting cycle valve tubing to c' the filtrate may be led to a measuring cylinder or other suitable receiver and it is certain that no small leak of the experimental liquid can pass into the filtrate.

Great trouble is experienced in summer time through flies attaching themselves to the drying collodion, so that a screen of muslin stretched on a wire frame should be provided.

A simple auxiliary device may be mentioned in passing. When say fifty cc. or less are to be handled the pressure bottle shown in Fig. 2 is obviously unsuitable. A piece of stout glass tube drawn out to a nipple at each end is clamped vertically close to the apparatus. The liquid to be forced into the apparatus may now be placed in this tube and the pressure bottle is simply used as a compressed air reservoir and is connected to the top of the tube.

Portion of the filtering area inoperative through presence of air bubbles.

The films are, of course, impervious to air and it may be thought that a certain quantity of filtering surface will not be utilised because the air in the passages will be forced on to the filter. Examination of the films, when the apparatus is taken to pieces after a filtration, shows that owing to the fineness of the passages the proportion of filtering area so rendered inoperative is very small, and may be entirely abolished if care is taken that all the channels are full of liquid before filtration commences. This being so it is a matter of indifference what the position of the apparatus is in use: in some experiments where it was desired to keep the temperature of filtration under control it was immersed horizontally in a large shallow basin: in others the bracket was screwed to the top of the table near the edge with the plates hanging down vertically.

Use of One Film only.

If one film only provides sufficient filtering area on any occasion it is convenient to put it between the *filter plate* and the *front plate*: the position of the other film between the *filter plate* and the *back plate* being occupied by a "dummy," an impervious film cut from rubber insertion. In order that the channel *da* may not be filled unnecessarily the filter plate should be put in back to front—the other channels *c* and *c'* which are symmetrically placed will of course be unaltered in position.

It is frequently desirable to recover the "ultraprecipitate," which is usually a thick gum, when the filtration is over. This may be done either by taking the apparatus to bits or by attaching a receiver to the nipple and aspirating. Washing out may be done directly by running water in and out. Except in so far as the films remain impregnated with the materials filtered off, they are recovered clean in this way, as the subsequent examination of the film plainly shows.

Although from the design of the apparatus it would appear at first sight as if the volume of fluid actually spread over the film at any one time was very small indeed, it is actually considerable, say 15 cc., and depends upon the pressure applied.

The film, of course, sags between the "pyramids" while filtration is in progress, though if the pressure is released, and still more so under suction, any remains of the liquid to be filtered are forced back through the nipple again.

Another and perhaps better way of going about the recovery of the "ultraprecipitate" is to screw in the nipple at a' and transfer the screw shown occupying a' to b. The liquid to be filtered is then pumped in at a'. The wash liquid is passed in and out at this point and b is used simply as an air vent.

Dialysis.

A simple procedure, which is frequently useful, for recovering a material, say a protein from a concentrated salt solution, may now be described by citing an actual example. The material, say a precipitate of crystalline egg albumin wet with ammonium sulphate solution, is to be handled with a view to obtaining the egg albumin in concentrated solution free from electrolyte. We will suppose again that only one filter is to be used. The precipitate is wetted with distilled water until fluid, and filled into a suitable glass tube drawn out at both ends (see page 258). Its lower end is attached to the nipple in b or a' and the liquid forced all over the film by air pressure from the bottle. Tap water and, later, distilled water are run across the filter plate, in at c and out at c', care being taken to get the air out of the grooves between the pyramids first. Owing to the high osmotic pressure of the ammonium sulphate originally present some of the fluid being dialysed may regurgitate into the tube but as the dialysis and ultrafiltration proceed the ammonium sulphate is carried away. In fact, it is generally possible to regulate the pressure so that the space between the front plate and the filter is just full, *i.e.* the whole of the dialysing surface is operative. The design of the apparatus ensures extremely rapid dialysis for the volume of the liquid under treatment per sq. cm. of dialysing surface is as small as it very well can be made.

When examination of either the liquid being dialysed, or of the other liquid, or of both, shows that removal of the ammonium sulphate is sufficiently complete, then the liquid may be run off the filter plate and increased pressure applied. The egg albumin may, at the conclusion of the resultant ultrafiltration, be recovered as above.

4″ Apparatus—Pattern B. Diameter of Filter 3″.

For small scale operations, such as experiments on the properties of the individual members of a series of films, or to determine which film of a series is most suited for a particular operation, the simple apparatus (Fig. 3), which may be screwed up in any ordinary vice or clamp, has been found of service.

It takes one film between two pieces of aluminium, one recessed and fitted with a piece of pyramid pattern material to act as a filter plate, and the other left flat. The discs, supported and protected by being backed by pieces of iron, are suitably drilled and tapped to take the nipples, and the edges between which the film is held are ground face to face after being turned true. The perfection of this joint enables even the hardest nitro-cellulose films to be used without any leak at pressures from 50–80 lbs. per sq. inch. The films must, however, be uniform and flat.

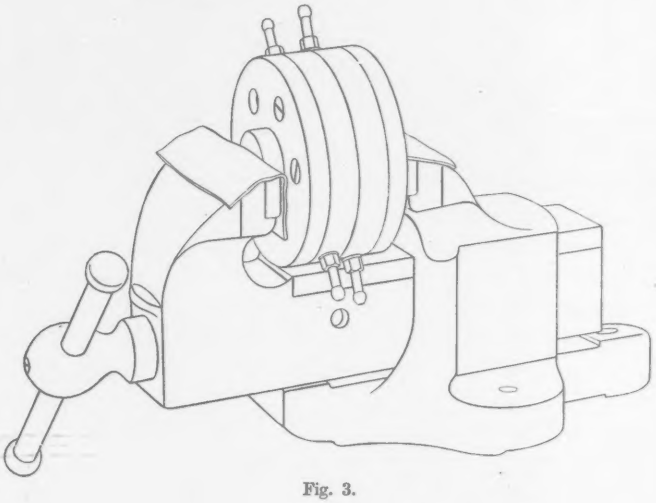

Fig. 3.

Separation of chloride from sulphate by dialysis.

I am able to confirm with flat films of nitro-cotton (British Xylonite Co.) Brown's observation [Brown, 1915] made with films of test-tube shape that this separation may be readily carried out. For assistance in obtaining suitable nitro-cotton to work on and ready advice and help in its use I am indebted and grateful to Dr J. N. Goldsmith. The following is the procedure that I have found satisfactory.

157 cc. of collodion (3·5 per cent. dry nitro-cotton in equal parts by volume of alcohol and ether) are poured on to a 12″ disc of levelled plate glass in the usual way. To prevent the drying film from curling off the glass the edge is painted round with collodion containing castor oil when half an hour has elapsed. Next day when the film is quite dry it is cut round with a penknife

using an 11½″ circular template. It is found that when perfectly dry it detaches itself readily from the plate—a uniform film which may be preserved indefinitely. Following Brown's directions this must be softened and made of the right degree of permeability by soaking in alcohol of the required strength. If a whole film is so treated the result serves admirably in the 12″ apparatus: but the small one does just as well on this occasion, so that from the original dry film four circles may be cut of ample size for this operation. Four trials may thus be made from one full-sized film. As a matter of fact twenty-four hours soaking in dilute alcohol, 50 % by volume, gives a film of the required properties. Such a film allows considerable quantities of chloride but no trace of sulphate to pass by dialysis against distilled water from a solution containing 1 % of sodium chloride and 1 % of sodium sulphate.

There have now been described two methods of preparing flat and uniform *standardised* ultrafilters of nitro-cotton and the question naturally arises as to which method is the more convenient and more reliable.

It is my opinion that where a "soft" filter is required—one, say, for separating an enzyme or a toxin from peptones, and other simpler substances —it is simpler to pour the film in the usual way and to plunge glass and all into water when drying has proceeded to the correct extent. On the other hand, where hard filters are required, and one must be prepared to differentiate, say, between those allowing dextrin to pass and those allowing the passage of sugar but not dextrin, Brown's method is certainly to be recommended.

REFERENCES.

Brown (1915). *Biochem. J.*, **9**, 591.
Walpole, S. G. (1915). *Biochem. J.*, **9**, 284.

XXIII. ON THE PRECURSOR OF CREATINE IN CHICK MUSCLE.

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(*Received May 16th, 1916.*)

In the search for the precursor of creatine and creatinine workers naturally turned to arginine and its disintegration products. According to the theories of Knoop [1910] and Neubauer [1911], arginine is oxidised in the animal organism to guanidine-butyric acid, then to glycocyamine. Glycocyamine is then methylated to form creatine. There is no doubt that from the oxidation of arginine-containing proteins, guanidine-butyric and guanidine-acetic acids and even guanidine itself are obtained [Kutscher, 1901]. The methylation of a compound, *in vivo*, has been carried out successfully by many investigators. It is, therefore, reasonable to presume that the administration of compounds containing a guanidine or methylamino radicle will produce alterations in the creatine-creatinine metabolism.

Glycocyamine. The earliest researches were made by Czernecki [1905] who added quantities of glycocyamine to the diet of rabbits, and thereafter estimated the urinary creatinine. Using the zinc chloride method he obtained, in some instances, an increased excretion. He, however, considered that his results were indecisive.

Jaffé [1906] and his pupil Dorner [1907] observed a transformation to creatinine of from 4·5 to 12·7 % of the glycocyamine given to rabbits by the mouth. They also obtained a considerable increase in the creatine content of muscle. Dorner, too, after a study of the effect of adding glycocyamine to autolysing muscle came to the conclusion that methylation of guanidine-acetic acid to creatine takes place.

Mellanby [1908] arrived at the opposite result. He added glycocyamine in suspension to the diet of chicks on each of five days, and then determined the creatine content of their muscle and also of the muscle of the same number of controls. He found less creatine in the glycocyamine-fed chicks than in the normal.

Recently, Palladin and Wallenburger [1914, 1915] have repeated Dorner's experiments on the addition of guanidine-acetic acid to autolysing muscle. They used rabbit and eel muscle and found a decided production of creatine. In injected rabbits methylation occurred invariably, producing an increase in creatine content of from 21·5 to 35·6 %.

It is unfair to dismiss the question of the possibility of the methylation of glycocyamine or its anhydride, glycocyamidine, in the organism, on the ground that these bases do not occur naturally [Barger, 1914] and that, therefore, the formation of creatine or creatinine from them is not a physiological process. A substance may occupy a very important position in intermediary metabolism and yet be totally undetectable. It may be an absolutely necessary link in a chain of chemical changes but because of the rapidity with which it is transformed into the next substance in series, it may only momentarily exist in a free state.

Arginine. Arginine is the only known constituent of protein which contains the guanidine grouping. It has, therefore, been suggested as a possible parent of creatine and creatinine.

Negative or indecisive results were obtained by Van Hoogenhuyze and Verploegh [1905], Jaffé [1906] and Thompson [1906]. The latter worker found that 73–96 % of the arginine given was excreted as urea. More recently, however, he [1913] was able to demonstrate an increase in the creatine content of the urine after the administration of this amino-acid to dogs and birds.

Positive results are recorded by Inouye [1912], Myers and Fine [1915] and Kutscher [1914]. On the contrary, Baumann and Marker [1915] could detect no increase in total creatinine when arginine was added to hashed muscle.

Kutscher [1914] thinks that the formation of arginine is an intermediate step in the formation of creatine. It may be so rapidly transformed into the latter that detection is impossible, but in the lower organisms, for example, crustacea, the chain of chemical changes may not go so far as creatine and so arginine be found. In mammalian muscle creatine is a constant constituent and arginine unknown, while in the crayfish Kutscher demonstrated the presence of arginine and the absence of creatine.

Other possible precursors. Sarcosine may yield creatine *in vitro* [Volhard 1868, cited by Barger 1914], and Riesser [1913] administered this amino-acid in conjunction with urea, *per os* and subcutaneously, and in half of his experiments got evidence of creatine formation.

Recently [1914] he put forward the hypothesis that creatine was formed as one of the by-products of lipoid metabolism. To confirm his theory he studied the influence of injections of choline and of betaine on the creatine content of rabbit muscle, and on the creatinine elimination in urine. Choline produced positive results in four out of seven experiments while betaine gave small increases every time. Methylureido-acetic acid was perfused through isolated limbs by Baumann and Marker [1915] but without result.

Baumann, Hines and Marker [1916] perfused arginine, betaine, methylureido-acetic acid, sarcosine plus urea, and choline plus urea through muscle tissue. Only choline plus urea consistently led to an increase in muscle creatine.

It seems natural to suppose that guanidine itself might be the precursor of creatine or creatinine. It could combine with acetic acid to form glycocyamine which could add a methyl group to form creatine, or guanidine might first be methylated and then combine with acetic acid. Guanidine (or methylguanidine) may exist in muscle in two states. It may either form part of the arginine molecule or, as suggested by Jaffé and Otori, it may be the chemical nucleus to which the various peptide chains are attached.

Evidence of the formation of creatine from guanidine or methylguanidine has not as yet been found. Success did not attend the efforts of Jaffé, Dorner, Pommerenig [1901] or Achelis [1906] who administered methylguanidine. Neither did Riesser [1914] who injected both guanidine and methylguanidine obtain any increase in the quantity of urinary creatine.

From a review of the literature on the subject of the origin of muscle creatine and its relationship to urinary creatinine, one must come to the conclusion that adequate facts are wanting on which to base a satisfactory theory of creatine metabolism.

Muscle of chick embryo contains no creatine up to the twelfth day of incubation. Does it contain guanidine?

In 1911, Dr Cathcart suggested to me that a study of the total guanidine content of fertilised eggs at various stages of development might throw some light on the formation of creatine. Mellanby [1908] has shown that fertilised hen's eggs contain no extractable creatine till the twelfth day of incubation, and then a trace only, which, however, increases in amount till the egg has hatched.

Kutscher [1901] suggested that possibly arginine may be the precursor of

creatine. Arginine may be oxidised *in vitro* to γ-guanidine-butyric acid. Through β-oxidation this would yield guanidine-acetic acid. Jaffé, Dorner, and others have demonstrated the methylation of guanidine-acetic acid to creatine.

Arginine	Guanidine-butyric acid	Guanidine-acetic acid	Creatine
NH_2	NH_2	NH_2	NH_2
$\dot{C}=NH$	$\dot{C}=NH$	$\dot{C}=NH$	$\dot{C}=NH$
$\dot{N}H$	$\dot{N}H$	$\dot{N}H$	$\dot{N}-CH_3$
$\dot{C}H_2$	$\dot{C}H_2$	$\dot{C}H_2$	$\dot{C}H_2$
$\dot{C}H_2$	$\dot{C}H_2$	$\dot{C}OOH$	$\dot{C}OOH$
$\dot{C}H_2$	$\dot{C}H_2$		
$\dot{C}HNH_2$	$\dot{C}OOH$		
$\dot{C}OOH$			

The attempts that have been made to cause a formation of creatine in the body through the administration of arginine, guanidine and methylguanidine have already been considered above.

Method.

The guanidine molecule is very resistant to oxidation. It was noted by Kutscher and his co-workers that a soluble protein, treated with successive small quantities of a boiling solution of a permanganate, yielded a complex, from which most of the products of oxidation had been driven off as volatile aldehydes, ammonia, carbon dioxide, etc. By the use of a suitable precipitant, an insoluble salt of guanidine could be obtained.

Choice of Precipitant. Guanidine is a strong monovalent base forming a series of crystalline salts with acids. Of the salts formed with mineral acids, the nitrate, $CH_5N_3 \cdot HNO_3$, is among the least soluble. It forms large plates melting at 214°.

The picrate, $CH_5N_3 \cdot C_6H_3O_7N_3$, and the picrolonate, $CH_5N_3 \cdot C_{10}H_8O_5N_4$, generally come down as amorphous precipitates, often needing repeated recrystallisation to obtain the characteristic crystals.

Guanidine picrate when pure forms irregular clusters of leaflets (Fig. 4, Plate IV). On slow heating they melt at 311–315°—on rapid heating up to 320°. Emich [1891] states that one part of the guanidine salt is soluble in 2630 parts of water at 9°. It is more soluble in hot water, slightly soluble in alcohol and very slightly soluble in ether. Dilute soda and other alkalis have apparently no effect on it while concentrated acid and alkali dissolve

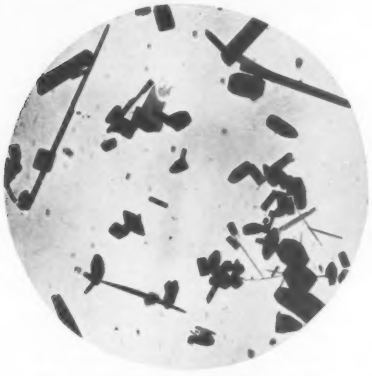


Fig. 1. Ammonium Picrate. ×50

Fig. 2. Ammonium Picrate from Egg. ×50

Fig. 3. Methylguanidine Picrate. ×50

Fig. 4. Guanidine Picrate. ×50

Fig. 5. Methylguanidine Picrate from Albumin. ×24

Fig. 6. Sodium Picrate. ×24

it rapidly. On heating on platinum foil it burns with a clear hissing flame leaving a coaly ash. It does not explode on heating or detonate when struck.

To precipitate guanidine from solution as picrate, the solution must not be too concentrated nor must it contain arginine [Kutscher and Otori, 1904].

Choice of Oxidising Agent. Calcium permanganate was chosen as oxidising agent, since the calcium can readily be removed as carbonate by the addition of the necessary quantity of sodium carbonate. After a trial of one or two "makes" of calcium permanganate, Merck's preparation was chosen as being most active and as being practically dry. In all the oxidations recorded below, a 10 % solution of Merck's calcium permanganate was the oxidising agent used.

Tests. Preliminary investigations were made on the guanidine content of gelatin, caseinogen, egg albumin and goose muscle.

(1) *Gelatin.*

Ten grams of commercial sheet gelatin (which gave a slight but distinct xanthoproteic reaction) were dissolved in 250 cc. water, and placed in a long-necked, round-bottomed Jena flask of one litre capacity, and the solution was brought to boiling point over an open flame. The solution of calcium permanganate was added, drop by drop, to this boiling fluid. The first drops produced a violent effervescence, and they were rapidly decolorised. As more permanganate solution was added, the effervescence became less marked, and the surface tension of the boiling fluid became greater, producing large bubbles. The boiling point of the semi-oxidised fluid was also raised. Finally, the surface tension returned to about its normal value, but each drop of the oxidising agent took a much longer time to become reduced.

The process of oxidation was stopped, either (*a*) when a certain quantity of calcium permanganate had been added, namely, 50 g.: or (*b*) when the last drop of permanganate was reduced only after prolonged boiling.

It will be seen that the oxidation process is divided into three periods.

(1) Early stage. This part is easily carried out and requires only occasional attention, for the purpose of regulating the rate of dropping of permanganate. There is a steady evolution of the aldehydes of the higher fatty acids, ammonia and carbon dioxide. Hydrocyanic acid was detected, and its presence confirmed by chemical means.

(2) Intermediate stage. Constant attention was necessary at this stage. Oxidative by-products were formed which raised the surface tension of the

fluid, and these had to be boiled off before another drop of the oxidising medium could be added. There was less smell of aldehyde and more of ammonia among the volatile by-products.

(3) Final stage. This stage presented no great difficulty. Its inception was sudden.

After cooling, the black manganese precipitate was filtered off on a Buchner funnel. The precipitate was transferred to the oxidising flask and lixiviated with about 250–300 cc. of hot water for six to eight hours, and was then cooled and filtered as before. This was repeated a second time. The filtrate and washings were united, evaporated on the steam bath to about 30–50 cc. and allowed to stand for twenty-four hours. The precipitate formed was then filtered off and washed with a small quantity of cold water. The filtrate was completely freed from calcium by the careful addition of a saturated solution of sodium carbonate, excess being avoided. The mixture was gently warmed, allowed to stand for about an hour, and then the calcium carbonate was filtered off. The filtrate which should be about 100 cc. was made up to that volume (approximately), and treated with a saturated solution of pure recrystallised sodium picrate. The guanidine picrate was allowed to stand in the mother liquor for twenty-four hours, filtered off, washed with a small quantity of cold water and then with ether. It was dried at 60° and weighed.

Results.

(*a*) Complete oxidation of 10 g. gelatin ...	1.	0·777 grams picrate	
	2.	0·772	,, ,,
	3.	0·773	,, ,,
(*b*) Oxidation with 50 g. $Ca(MnO_4)_2$ ...	4.	0·401	,, ,,

Two estimations of nitrogen were made of the picrate obtained from gelatin (2) and (3) (combustion process).

N of (2) 29·3; of (3) 30 %.

The picrate from gelatin (4) yielded 29·09 % of N.

Guanidine picrate contains 29·16 % nitrogen.

The substance did not melt below 280°. It was identified microscopically as guanidine picrate. (See later under "eggs.")

From the work of Kossel and Kutscher, we know that pure gelatin contains 9 % of arginine. Kutscher and Zickgraf [1903] consider that 10 g. of commercial gelatin correspond to 8 g. of the absolutely dry pure substance. Theoretically, 8 g. of pure gelatin should yield 1·19 g. of guanidine picrate.

The above workers actually got 0·75 g. from 10 g. of commercial sheet gelatin, that is 63 % of the theoretical yield. Our figures are sufficient to show that, under standard conditions, the method used is a comparative one for gelatin.

(2) *Application of the method to substances which are insoluble in water.*

Two methods are available for getting the insoluble protein material into solution, viz.:

I. Digestion with a proteoclastic enzyme.

II. Hydrolysis with a hot mineral acid.

Tests were carried out on commercial gelatin by both methods.

I. (*a*) 10 g. of gelatin were digested with 250 cc. of a 0·4 % solution of sulphuric acid, to which a small quantity of pepsin was added. The digestion lasted five days at 40°.

I. (*b*) The same quantity of gelatin was digested for the same time with 250 cc. of a 0·2 % solution of sodium carbonate, to which some pancreatin had been added.

II. The same quantity of gelatin was hydrolysed for nineteen hours with 250 cc. of 25 % sulphuric acid.

I (*a*) and II were then almost neutralised by the addition of less than the calculated quantity of barium carbonate, and were then oxidised as above, without the removal of the barium sulphate. I (*b*) was oxidised as it was.

Results.

A. *Complete oxidation.*

Peptic digest ...	0·6348 g.	guanidine picrate
Tryptic digest ...	0·23 „	„
Acid hydrolysis	0·834 „	„

B. *Oxidised with 50 g. permanganate.*

Peptic digest ...	0·4446 g.	guanidine picrate
Acid hydrolysis	0·4459 „	„

It will be seen that oxidation after acid hydrolysis is productive of a greater yield of guanidine than after the substance has been brought into simple solution with water, or by means of a peptic digest. This is in accordance with the results of Kunze [1910] and Henzerling [1910] who found that an alkylated creatinine gave (on oxidation) a better yield of a substituted guanidine if it had previously been hydrolysed with acid than if it had been digested with pepsin or trypsin or both. Apparently, the enzymic splitting of the molecule differs from that produced by acid hydrolysis.

(3) *Caseinogen.*

Several attempts were made to isolate guanidine from the oxidation products of commercial caseinogen.

Two experiments using a peptic digest, and two using a pancreatin solution, were made. In all cases a paranuclein was formed. Twice, caseinogen (10 g.) was hydrolysed for twenty hours with sulphuric acid and then, after being almost neutralised, was oxidised with calcium permanganate. In no case was any guanidine found.

30 g. of caseinogen were shaken up with 300 cc. of a dilute acetic acid solution and then oxidised direct. No guanidine was isolated.

According to Hart [1901] pure caseinogen contains 4·8 % of arginine and therefore should yield about 1 % guanidine. The commercial commodity probably contains less. It follows that the method detailed above is not suitable for the isolation of small quantities of guanidine from large amounts of material.

(4) *Muscle.*

An attempt was made to determine the guanidine content of goose muscle.

I. 10 g. of minced goose muscle (moist) were digested for seven days at 40° with pepsin and hydrochloric acid, as above.

II. The same quantity of goose muscle was similarly treated with pancreatin solution.

They were both completely oxidised, as above. The peptic digest required almost 20 g. and the pancreatic digest 16 g. of permanganate, for complete oxidation.

Results.

I. Peptic digest of goose muscle gave 1·72 g. of guanidine picrate

II. Tryptic „ „ „ 1·94[1] „ „

That is, moist goose muscle yields from 0·34–0·39 % guanidine.

Nitrogen in the picrate by combustion. I = 29·1 %. II = 24·7 %.

Dr Cathcart took samples of the above muscle, and, by the method described by Brown and Cathcart [1909], estimated the creatine content.

I. Leg muscle, 0·387 % creatine

II. „ 0·393 „

Creatine contains 45·7 % guanidine. That is 0·17 and 0·18 respectively should be subtracted from the guanidine percentage given for muscle in order to obtain a figure for the non-creatine guanidine. Muscle, therefore, contains

[1] This was almost entirely methylguanidine picrate contaminated with a small amount of sodium picrate.

some other source of guanidine than creatine as it yields from 0·17–0·2 % non-creatine guanidine.

(5) *Deaminised protein.*

In the process of auto-deaminisation albumins are altered beyond the stage of amino-acids. Kutscher [1901] failed to obtain arginine from auto-digested thymus.

Reaction with either nitrous acid, or with an alkali nitrite and acetic acid, is supposed to destroy completely the $CO \cdot NH_2$-group of the protein while it does not alter the protein in such a way as to prevent the biuret reaction. It has been shown that a deaminised protein may be hydrolysed into products containing NH_2-groups.

In order to ascertain whether deaminised gelatin or caseinogen contained guanidine, 50 g. commercial gelatin were thoroughly shaken on an electric shaker with 500 cc. of 10 % acetic acid. The resultant mixture was transferred to a large basin, and was warmed to less than 40° on a water bath. A 10 % solution of sodium nitrite was added drop by drop to the well stirred liquid, till a drop no longer produced foaming. The precipitate was filtered off, washed with water and then with alcohol, and finally with ether, and dried *in vacuo*.

10 g. at a time of this deaminised gelatin were hydrolysed, and completely oxidised, as above. No guanidine picrate was obtained (two expts.). In the same way, deaminised caseinogen was prepared and oxidised. Guanidine was not obtained.

Therefore, from the gelatin experiments, one may conclude that nitrous acid destroys the guanidine radicle and also the arginine molecule. Gergens and Baumann [1876] and Pommerenig [1901] demonstrated that both guanidine and methylguanidine underwent little or no deaminisation in the animal body. It is, therefore, probable that guanidine, as such, does not exist in muscle, except perhaps in the merest traces.

(6) *Pure Egg Albumin.*

4 g. of Merck's Egg Albumin (powder) were hydrolysed and oxidised as above.

Results.

(1) 0·254 g. of the picrate. (Nitrogen 28·7 %.)
(2) 0·288 „ „ („ 27·8 %.)
(3) 0·238 „ „ („ 27·9 %.)

Microscopic examination showed that the bulk of the yield was methylguanidine picrate. No. (2) contained a very small quantity of sodium picrate.

Guanidine hydrochloride ($CH_5N_3HCl \cdot H_2O$) should yield 253 % of the picrate.

0·2 g. of the hydrochloride was boiled up with acid and permanganate as above. 0·512 g. of the picrate was obtained (nitrogen 27·5 %); this contained traces of the precipitant.

0·2 g. of guanidine hydrochloride + 4 g. of egg albumin powder were hydrolysed, neutralised and oxidised as before. Yield, 0·875 g. of the mixed picrates of guanidine and methylguanidine.

(7) *Eggs.*

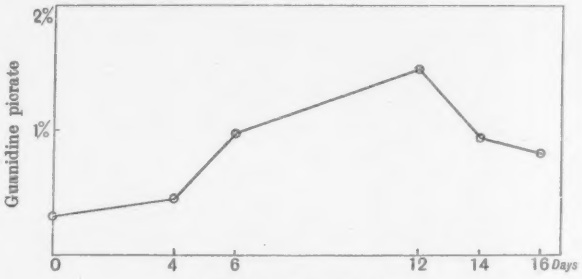
The fertilised eggs, on arrival, were carefully repacked, and allowed to stand for twenty-four hours. They were then placed in the laboratory incubator kept at 40°, the air being saturated with moisture at that temperature. Each day, at the same hour, the eggs were turned by hand. At certain periods, indicated below, two eggs (Set I only) were removed from the incubator and their weight and the weight of their shell taken. The embryo was carefully examined as to development, and if its age, thus determined, did not agree with that calculated from the number of days spent in the incubator, it was at once rejected. (I am much indebted to Dr W. Abel—Mrs Shaw Dunn—for her kind help in adjudicating ages.) The embryo was then transferred to the oxidising flask and roughly extracted with ether. This removed most of the fatty matter of the yolk (triolein and tristearin), the colouring oil (chromatin) as well as cholesterol and ovine. The resultant matter was hydrolysed with 250 cc. of 25 % sulphuric acid for twenty hours, almost neutralised by the addition of 140 g. of barium carbonate, and oxidised with 50 g. of calcium permanganate, as above. That the results of this tedious process are at least comparative is shown by the similarity of the figure obtained for eggs of the same age. For example, the amount of guanidine in an egg incubated for fourteen days is given (Set I) as 0·47 and 0·44 g. of the picrate. If this is calculated as the amount of the base per 100 g. of egg substance, the figures are even closer, viz.: 0·19 % and 0·188 %. For eggs which had been incubated for about two and a half days, one gets (Set II) 0·24 % and 0·236 % of guanidine. Eggs of different sets are also comparable age for age. From Sets II, III and IV, results may be obtained for eggs of three days' incubation, namely, 0·298 %, 0·3 % and 0·3 % guanidine. The results are tabulated below, and are also presented graphically, Set I in Graph 1, and the others in Graph 2. The first series is not strictly comparable with the later sets, as the process was slightly improved in detail.

It was found to be useless to carry out estimations of the N content

of the picrates as the yields consisted of mixtures of guanidine and methyl-guanidine. Microscopic analyses had to be taken as the sole criteria of composition, and photomicrographs of various substances met with are given in Plate IV.

Results. *Set I. May*, 1911.

Days incubated	Weight of egg g.	Weight of shell g.	Weight of material taken g.	Guanidine picrate g.	% Guanidine picrate	% Guanidine (base)
0	68·7	8·3	60·4	0·1828	0·30	0·06
4	56·6	5·84	50·76	0·231	0·45	0·09
6+	60·9	6·9	54	0·5328	0·98	0·196
12	60·45	6·75	53·7	0·779	1·45	0·29
14	56·9	7·7	49·2	0·4707	0·95	0·19
14	54·35	7·15	47·2	0·44	0·94	0·188
16	59·52	7·75	51·77	0·4295	0·82	0·164



Graph 1. Eggs—Set I

In Sets II, III and IV slight alterations were made in the method of analysis. For instance, the barium sulphate, formed on neutralising the products of acid hydrolysis, was filtered off, and was extracted three times by boiling with water. Further, oxidation was carried out more slowly. Care was taken that as much as possible of the volatile products of oxidation was driven off by boiling before any additional permanganate was dropped in. The same quantity of permanganate was used—50 g. The final liquid was reduced further in bulk, before the precipitation of guanidine by sodium picrate.

Set II. Incubation started March 11, 1912.

Date	Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
20/3/12	48 hrs.	56·6	6·3	50·3	0·6126	1·2	0·24
23/3/12	56 hrs.	59·0	6·95	52·05	0·636	1·2	0·24
,,	,,	57·4	7·47	49·93	0·5835	1·18	0·236
22/3/12	3 days	57·25	6·05	51·2	0·7656	1·40	0·28
,,	,,	55·45	6·62	48·83	0·7759	1·5	0·3
21/3/12	4 days	56·27	6·7	49·57	0·9005	1·82	0·364

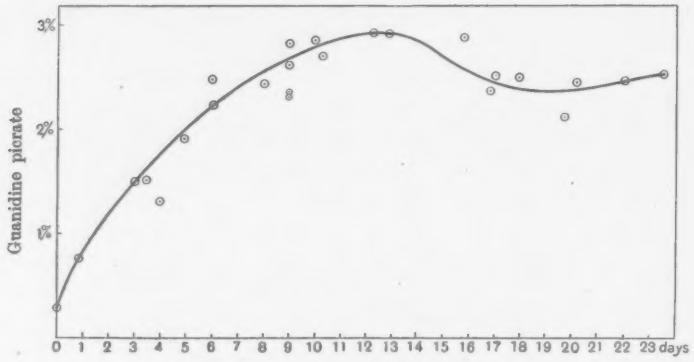
Set III. Incubation started May 31, 1912.

Date	Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
June 3	16 hrs.	60·05	5·9	54·15	0·4315	0·79	0·16
„ 4	3 days	54·95	5·85	49·1	0·76	1·5	0·3
„ 5	4 „	52·3	6·25	46·05	0·586	1·3	0·26
„ 7	6 „	56·09	6·59	50·05	1·2727	2·5	0·5
„ 10	9 „	53·61	6·4	47·21	1·3593	2·8	0·56
„ 10[1]	8 „	47·69	5·96	41·73	1·3076	3·1	0·62

[1] June 10, 1912. Age 8, had died the day before removal. Guanidine picrate contaminated with larger reddish crystals of ammonium picrate (Fig. 2, Plate IV).

Set IV. Incubation started June 25, 1912.

Date	Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
July 4	9 days	53·3	6	47·3	1·1603	2·6	0·52
„ 5	3 or 4 days (dead)	53·6	6	47·6	0·6951	1·5	0·3



Graph 2. Sets II, III, IV and V

The incubation of the eggs in the laboratory incubation chamber had to be abandoned as it was difficult to keep the eggs alive more than nine days.

The following was the death rate in the earlier sets.

Set I.	6	died	before	the ninth day.
„ II.	8	„	„	„
„ III.	7	„	„	„
„ IV.	11	„	„	„

That is, over 66 % of the eggs had to be rejected as undeveloped.

Arrangements were then made with Mr A. B. Templeton, the proprietor of the "Sussex Hatcheries," to forward eggs to the laboratory as required. The eggs were removed from his incubator at 8.30 a.m., placed in a receptacle

designed to retain warmth, and sent by special messenger to the laboratory where they were at once weighed, examined and submitted to acid hydrolysis.

In this series the removal of ether-soluble bodies was omitted to save time. The actual result, however, of the omission was to cause additional trouble. The presence of oily matter should, of course, have kept the surface tension of the fluid low. The oxidation products of fats seem to have the opposite effect. The second stage of the oxidation process was marked by a particularly high surface tension which produced intense frothing. This necessitated careful heating often over several days and so the process was

Set V. Incubated at Sussex Hatcheries.

Age	Weight (total)	Weight (shell)	Weight (egg)	Guanidine picrate	% Guanidine picrate	% Guanidine (base)
5 days	58·8	5·915	52·885	1·0128	1·91	0·38
8 ,,	52·6	4·85	47·75	1·1666	2·44	0·49
9 ,,	53·3	5·9	47·4	1·1062	2·33	0·466
9 ,,	49·7	6·0	43·7	1·0123	2·3	0·46
10 ,,	52·67	6·5	46·17	1·3068	2·83	0·566
10 ,,	51·5	6·3	45·2	1·1881	2·70	0·54
11 ,,	53·03	6·01	47·02	1·1744	2·50 (error)	0·50
12 ,,	49·92	6·32	43·6	1·2956	2·94	0·59
13 ,,	49·95	5·6	44·35	1·3013	2·93	0·586
14 ,,	56·1	6·4	49·7	0·9975	2·01[1]	0·42
15 ,,	55·22	5·99	49·23	1·3297	2·70	0·54
16 ,,	53·25	5·78	47·47	1·3756	2·89	0·58
16 ,,	45·5	5·25	40·25	0·7832	1·94[1]	0·39
17 ,,	50·95	5·99	44·96	1·0692	2·37	0·474
18 ,,	52·65	6·9	45·75	1·166	2·5	0·5
19·75 ,,	52·0	6·65	45·35	0·961	2·11	0·42
20 ,,	49·6	5·7	43·9	1·0691	2·43	0·486
22 ,,	—	—	39·1	0·9754	2·49	0·498
23·5 ,,	—	—	34·8	0·8886	2·55	0·51

[1] Accidentally allowed to evaporate to dryness after filtration from Mn. ppt.

considerably lengthened. The volatile products of oxidation were particularly odorous. In addition to hydrocyanic acid and the other bodies already mentioned, butyric aldehyde and acid were noticed.

Great differences were noted in the difficulty of oxidising different eggs. Those at both ends of the series were fairly easy to work with—*i.e.* up to eight days and after sixteen days—while it was with great difficulty that the eggs in the middle of the series were oxidised.

Egg No. 18—age ten days—was particularly refractory. About half way through the oxidation, that is when about 25 g. of permanganate had been added, a very high surface tension was developed. The last 10 g. of

permanganate were added with great difficulty. Each drop caused a persistent frothing which subsided after prolonged boiling. At times, the oxidising agent was being added at the rate of one drop in thirty minutes.

Before adding sodium picrate, precautions were taken to remove any ammonia present in solution. After the removal of the guanidine picrate formed, the mother liquor was evaporated down and allowed to stand. In most cases, a crop of crystals of the picrates of the mixed methylguanidines, was obtained. On further reduction of volume, ammonium picrate crystallised out in small amount.

The results of Sets II to V are plotted in Graph 2.

Discussion of Results.

The seven eggs in Set I show an increase in total guanidine content from 0·06 % of the unincubated egg to 0·29 % of the twelfth day of incubation. Thereafter, the percentage amount decreases rapidly to the fourteenth day and more slowly to the sixteenth.

The thirty-one eggs forming the other series represent ages from a one day egg to a two and a half day old chick (unfed). The results are plotted on the second graph, and it will be seen that both curves reach their maximum at the same point, namely the twelfth day. There is, as before, a steady increase in the amount of guanidine present from 0·16 % to 0·59 %. The decline after the twelfth day is not as regular as the incline before it. Just before the maximum is reached, there is a slight oscillation in value. On the ninth day, for instance, a slight dip occurs on the curve. If the curve were to remain smooth, there should be 0·56 % guanidine in the egg on that day. The actual figures are 0·5 and 0·46 %. The eleventh day embryo contains much less guanidine than one would expect from a study of the content of the tenth and twelfth day embryos. The filtrate from this guanidine precipitate contained a larger quantity of ammonium picrate than usual. Similarly, the 14 and 16·5 day old eggs are poorer in guanidine than one would naturally expect.

The first question that arises is, what is the cause of the twelfth day maximum?

(1) Mellanby [1908] has shown that, at that stage of development, creatine makes its first appearance as an extractable constituent of egg bioplasm. One might, therefore, consider that guanidine or its methyl derivative is continuously formed till its concentration is about 0·3 % of the

total weight of the egg. At that point, a rupture of the guanidine-containing molecule takes place with the formation of methylguanidine-acetic acid. This formation of creatine, however, does not seem to me to account for the subsequent decrease in the total guanidine content of the egg. The presence of creatine, if synthetic, might increase but in no way could decrease the amount of guanidine. It has been demonstrated that both creatine and its anhydride are readily oxidised, by the process used above, to guanidine and methylguanidine.

(2) Neither can it be that an equilibrium has been established between guanidine and creatine, for then after the twelfth day till hatching, the total guanidine would remain constant.

(3) This would also be the result if the complex containing guanidine ceased to be formed.

Two other possible explanations present themselves.

(4) The configuration of the protein complex may have been altered so that the new complex is more resistant to oxidation.

(5) It has been shown above that enzymes may split the protein complex through the guanidine molecule, so that the product gives a lower yield of guanidine on subsequent oxidation (page 269). From the work detailed above (page 271) on the action of nitrous acid on the guanidine content of gelatin, one concludes that the guanidine group (in arginine) remains free in the linking together of amino-acids to form the protein complex. This is shown in the diagram of the protein molecule given by Plimmer [1913, p. 10]. The position of arginine renders it open to the attack of enzymes, especially trypsin. Something of this nature may take place with the result that the precursor of guanidine which has not been converted into creatine is partially destroyed.

I fail to understand Mellanby's reasoning when he correlates the amount of creatine present in chick embryos of different ages with the development of the liver. There seems to be no striking alteration in either the weight or the activity of this organ on the twelfth day of incubation.

Further Noël Paton has clearly demonstrated the absence of any direct connection between the liver and creatine metabolism in the bird.

Important changes preparatory to hatching take place on the seventeenth to the nineteenth days. This is shown embryologically by the total absorption of the amniotic fluid, the retraction of the intestine and of the yolk-sac into the body cavity and the position of the head. Mellanby's curves—body weight, liver, and creatine—all show a flattening at this stage indicating an arrest in development during the period of rearrangement. The amount of

guanidine also varies irregularly during those days. There is primarily a rise followed by a slight drop. On the twentieth day a steady increase begins, lasting, at least, until two and a half days after hatching.

What importance may be attached to this arrested development and oscillation of the total guanidine content just before hatching can not yet be discussed.

Very little can be said at present of the relative proportions of guanidine and the various methylguanidines obtained from developing eggs. The ease with which methylation takes place has already been alluded to, and one cannot but fear that much of the methyl derivative obtained is artefact.

Before any very decisive opinion could be expressed on the relation of guanidine to creatine formation in the developing egg, further work is necessary. When opportunity offers, a study of the free (extractable) guanidine as apart from that combined in protein or in creatine is to be carried out.

Summary.

1. In the developing chick embryo there is a regular increase of the guanidine content till the twelfth day of incubation.

2. Thereafter there is a marked decrease, followed by a period of oscillation and finally a slight but steady rise.

This research was begun under the tenure of a Carnegie Scholarship, and continued by the aid of an annual grant from the Carnegie Trustees to whom I am much indebted. I am also under a debt of gratitude to Professors Noël Paton and E. P. Cathcart for their helpful guidance and kindly criticism.

REFERENCES.

Achelis (1906). *Zeitsch. physiol. Chem.*, **50**, 10.
Barger (1914). *The Simpler Natural Bases*, p. 78.
Baumann and Marker (1915). *J. Biol. Chem.*, **22**, 49.
Baumann, Hines and Marker (1916). *J. Biol. Chem.*, **24**, *Proc.* xxiii.
Brown and Cathcart (1909). *Biochem. J.*, **4**, 420.
Czernecki (1905). *Zeitsch. physiol. Chem.*, **44**, 294.
Dorner (1907). *Zeitsch. physiol. Chem.*, **52**, 225.
Emich (1891). *Monatsh.*, **12**, 23.
Gergens and Baumann (1876). *Pflüger's Arch.*, **12**, 205.
Hart (1901). *Zeitsch. physiol. Chem.*, **33**, 347.
Henzerling (1910). *Arch. Pharm.*, **248**, 604.
van Hoogenhuyze and Verploegh (1905). *Zeitsch. physiol. Chem.*, **16**, 169.
Inouye (1912). *Zeitsch. physiol. Chem.*, **81**, 71.
Jaffé (1906). *Zeitsch. physiol. Chem.*, **48**, 430.
Knoop (1910). *Zeitsch. physiol. Chem.*, **67**, 495.

Kunze (1910). *Arch. Pharm.*, **248**, 578.
Kutscher (1901). *Zeitsch. physiol. Chem.*, **32**, 415.
—— (1914). *Zeitsch. Biol.*, **64**, 240.
Kutscher and Benech (1901). *Zeitsch. physiol. Chem.*, **32**, 278.
Kutscher and Otori (1904). *Zeitsch. physiol. Chem.*, **43**, 92.
Kutscher and Zickgraf (1903). *Sitzber. Akad. Berlin*, 1.
Mellanby (1908). *J. Physiol.*, **36**, 447.
Myers and Fine (1915). *J. Biol. Chem.*, **21**, 389.
Neubauer (1911). *Abderhalden's Handlexicon d. Biochemie*, **4**, 386.
Palladin and Wallenburger (1914). *Bull. Acad. Imp. Sciences Petrograd*, 1427.
—— —— (1915). *Compt. Rend. Soc. Biol.*, **78**, 111.
Plimmer (1913). *The Chemical Constitution of the Proteins.* Part II.
Pommerenig (1901). *Beiträge*, **1**, 561.
Riesser (1913). *Zeitsch. physiol. Chem.*, **86**, 415.
—— (1914). *Zeitsch. physiol. Chem.*, **90**, 221.
Thompson (1906). *J. Physiol.*, **33**, 106.
—— (1913). *Inter. Congress of Med.* (Physiol. Section).

XXIV. ON THE CONCENTRATION OF ANTITOXIC SERA.

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(*Received May 22nd, 1916.*)

Of the various methods which have from time to time been proposed for the concentration of sera, those developed by Banzhaf have been most widely adopted on a commercial scale. In this communication it is only necessary to refer to:

A. *The Banzhaf-Gibson* [1907] *Two Fraction Method,* in which serum or plasma, diluted with one-third its volume of water, was heated to a temperature of 57° for 15 hours, or of 58° for 7 hours. The liquid was then made 30 per cent. of saturation with ammonium sulphate[1] and the ensuing precipitate (Fraction I) separated by filtration.

The Fraction I precipitate was suspended in saturated brine solution for 48 hours or longer. The liquid was filtered from the insoluble euglobulin and the filtrate, containing in solution the pseudoglobulin and antitoxin which had been present in the Fraction I precipitate, was made 0·25 per cent. with glacial acetic acid. The ensuing precipitate was filtered, pressed and dialysed.

The filtrate from Fraction I was made 54 per cent. of saturation with ammonium sulphate and the precipitate (Fraction II) thus formed was filtered, pressed and dialysed.

The dialysis residues from Fractions I and II were mixed, the requisite amount of salt and preservative added, and finally filtered ready for distribution. This method has been used in these laboratories for some considerable time and it has been found that by this process a good end product can be

[1] Throughout this paper the phrase "made x per cent of saturation with ammonium sulphate" has been used to indicate the following: To $100-x$ parts of the serum by volume there were added x parts by volume of a saturated aqueous solution of ammonium sulphate.

obtained which remains clear for years. Care must, however, be taken in the first precipitation, as otherwise some of the insoluble globulin passes on and appears in the final product which, in this case, does not remain clear.

B. *Banzhaf's* [1913] *One Fraction Method,* in which serum or plasma, diluted with half its volume of water and made 30 per cent. of saturation with ammonium sulphate, is brought up to a temperature of 60°, the entire heating process taking about two hours.

According to Banzhaf the filtration of the hot serum mixture should proceed rapidly. The precipitate of euglobulin is pressed well or is washed with 33 per cent. saturated ammonium sulphate; the washings are filtered and added to the main bulk of the filtrate which is then made 50 per cent. of saturation with ammonium sulphate. The ensuing precipitate, consisting of pseudoglobulin and antitoxin, is filtered, pressed, dialysed, and the dialysis residues treated in the same way as in the Banzhaf-Gibson process.

Banzhaf considers that, during the shortened heating of the mixture in the new method, a certain amount of pseudoglobulin is converted into euglobulin, and bases this conclusion on his own experiments [1908]. The effect of heating the serum mixtures is thus to ensure the precipitation of an amount of euglobulin which ordinarily would require a higher percentage of ammonium sulphate for precipitation, and which does not, as would happen in the latter case, carry down with it an appreciable amount of pseudoglobulin and antitoxin.

Banzhaf claims that the new process possesses the following advantages over the former Banzhaf-Gibson process.

(1) There is only one finished product for dialysis.

(2) There is a considerable saving of time for the reason that the first precipitate (30 per cent. of saturation with ammonium sulphate), instead of being subjected to the more lengthy process of extraction with saturated brine and subsequent precipitation of the brine extract with acetic acid, is washed with 33 per cent. ammonium sulphate and the washings added to the main filtrate.

(3) The expenses incurred in the process are considerably less, both as regards labour and the use of precipitating materials, etc.

This new method certainly possesses the advantage of being a much shorter process than the former Banzhaf-Gibson method, but unfortunately it is not always easy to work. The difficulty that sometimes arises in carrying out the process is that the serum will not filter well. This difficulty has arisen in the course of my work both in an English and in a Canadian laboratory; in

the latter laboratory I had the advantage of consulting Dr Banzhaf personally about the matter. He suggested that a slight alteration in temperature or in the concentration of ammonium sulphate might be tried to overcome the difficulty, but these suggestions, although of value in some cases, did not always provide a satisfactory solution to the problem. The majority of workers on the subject with whom I have discussed the question have been faced with the same irregularity in concentrations by Banzhaf's new method.

Practical experience shows that the production of a readily filterable end product which will remain almost permanently clear, goes hand in hand with the rapid filtration of the serum mixtures after the preliminary heating process. When the heated mixtures filter with difficulty it is in some cases necessary to allow the dialysed pseudoglobulin-antitoxin precipitate to stand in the ice chest for weeks in order that the opalescent suspension therein may settle—otherwise filtration through filter candles is accomplished with great difficulty and, moreover, the final product will not remain clear.

This delay is a great disadvantage, and is one which nullifies any gain in time which should accrue from the fact that there is only one fraction to be considered.

It was obvious that the technique of the new process called for improvement or for enlargement, and in this laboratory we have ascertained that the above mentioned irregularities in the filtration can be obviated by the addition of sodium chloride to the serum mixtures, as described below.

The serum or plasma is diluted with one-half its volume of water, made 30 or 31 per cent. of saturation with ammonium sulphate and to the mixture is added 1·5 per cent. of solid sodium chloride. The temperature of the mixture is gradually raised to 61°, 63° or even 65° and kept at this point for a few minutes only. The mixture is allowed to cool to 40°–45° and filtered.

In every case the filtration of this hot mixture and of the subsequent 50 per cent. saturation proceeds rapidly. The liquid obtained after dialysis of the pseudoglobulin-antitoxin precipitate shows no sign of the presence of an opalescent colloidal suspension. After filtration through muslin it is as clear to the naked eye as though it had already been passed through paper pulp. On standing in the ice box these dialysis residues remain clear as there is nothing in opalescent suspension to be deposited.

On the addition of cresylic acid or tricresol to the dialysis residues a certain amount of precipitate is formed in all cases. In the batches of concentrated serum to which the 1·5 per cent. of solid sodium chloride has been

added this deposit is exceedingly slight, even after the serum has stood many weeks. Such a serum may undergo its final filtration through pulp and filter candles almost at once: the end product will remain perfectly clear.

By the addition of 1·5 per cent. solid sodium chloride to the serum mixtures we have so far obtained a satisfactory end product with certainty and the loss of antitoxic units has been very small; a most important item to be considered in assessing the value of any method. From incomplete experiments we have reason to believe that by a further study of the dilution of the plasma, of the concentration of the ammonium sulphate and of sodium chloride and of the general treatment of the mixture, we may be able to reduce the loss of antitoxin to a minimum and at the same time produce a much better end product.

At Dr MacConkey's suggestion, an effort has been made to control the heating of the serum by means of the immersion refractometer, in order to see if some easy indication might be obtained as to the best point at which to interrupt the process. This appeared hopeful in view of Banzhaf's explanation of the effect of heating. However, the results obtained from the use of this instrument, of which the reliability and accuracy have been well established, have demonstrated that no useful indication was forthcoming, for, contrary to Banzhaf's hypothesis, during the heating process there is no appreciable change in the amount of protein in solution in the serum mixture. This result, not entirely unsuspected, has led to some interesting investigations which have been carried out with routine and with experimental batches of serum in order to elucidate:

I. The value of the heating process in the new One Fraction Method.

II. The part played by the sodium chloride in improving the technique of Banzhaf's new process.

I. *The value of the heating process in the Banzhaf new One Fraction Method.*

As previously stated, it was originally hoped that a study of the refractive indices of the filtrates of samples taken from the serum mixtures from time to time during the heating process would indicate the point at which optimum conditions for filtration had been reached. With this object in view the following procedure was adopted.

Routine concentrations were carried out in which serum or plasma, diluted with one-half its volume of water, and made 30 per cent. of saturation with ammonium sulphate, was heated to a temperature of 61°, 63°, 65° and

kept at the specified temperature for a few minutes only, the process taking from 2 to 3 hours in all. Samples of the original plasma and of the plasma mixtures at various stages during the heating process were taken. The samples were filtered and the refractive indices of the clear filtrates were measured by means of the Zeiss Immersion Refractometer. In each case the clear liquid, thus examined, was boiled and filtered and the refractive index of the second filtrate which contained the serum salts and ammonium sulphate in solution, was noted. The amount of protein in solution in the serum mixture was calculated from the difference between the values of the refractive index of the whole serum mixture and of the filtrate containing the serum salts.

TABLE I.

Oxalated plasma diluted with half its volume of water and made 31 *per cent. of saturation with ammonium sulphate. The mixture was heated to* 63° *and kept at that temperature for about five minutes.*

Protein Content of the Original Plasma = 7·45 per cent.

Duration of time of heating of plasma mixtures	Temperature of mixtures	Scale Divisions of Refractometer Measurements of: (*a*) The filtrates from the mixtures	(*b*) The filtrates (*a*) diluted with an equal volume of water, boiled and filtered	Per cent. of protein remaining in solution expressed per 100 cc. of the original plasma
0	16°	98·65	51·50	5·11
1 hr 50′	53·5°	98·72	51·50	5·15
2 hrs 5′	57°	98·55	51·40	5·16
2 hrs 20′	59°	98·56	51·50	5·16
2 hrs 40′	61°	98·50	51·60	5·09
2 hrs 45′	62°	98·85	51·70	5·11
2 hrs 55′	63°	98·90	51·60	5·13

These results have been calculated from the values given in Reiss' table [1904]. For purposes of comparison it has been assumed throughout this research that in serum mixtures containing from 0 to 4 per cent. of protein the difference in protein content for 1 scale division = 0·220 per cent.; from 4 to 8 per cent. the difference for 1 scale division = 0·2175 per cent.

Brailsford Robertson [1915] considers that Reiss' figures are somewhat high, but since in the present investigation the *relative change* only in the protein content of the serum mixture is to be investigated it follows that it is quite immaterial whether Reiss' values are above or below the true value.

An examination of the results from a typical concentration given in Table I shows that during the heating process there was no appreciable change in the refractive indices of filtrates of the serum mixtures heated from room temperature to 63° or 65° and kept at these latter temperatures for

5 or 10 minutes. The very slight changes observed may have been due to the effect of heating the serum itself (*post* p. 301 and Table XIV).

Banzhaf seems to consider that the value of the heating lies in the conversion of soluble pseudoglobulin into a further amount of euglobulin, and that the rapid filtration of the serum mixture depends upon the success of this conversion. Had there been any such change in the amount of protein remaining in solution in the serum mixture, there should have been corresponding variations in the refractive indices of the solution. But, as no change occurred in this physical property of the mixtures one must conclude that a certain amount of euglobulin is thrown out of solution on the addition of the ammonium sulphate at room temperature, and that this amount is not further increased during the heating process.

Since the conversion of pseudoglobulin to euglobulin apparently does not occur during the heating process, it is not improbable that the value of the heating lies in its complete aggregation of the particles of precipitated euglobulin into conglomerates of such dimensions that they are readily retained on the surface of filter paper. The advantage of rapid over slow preliminary heating of the serum mixture—*i.e.* up to 57°—in producing an easy filtering fluid, is most readily explained on the same ground. If the heating were insufficient the smallest particles would pass the filter, while the slightly larger would actually engage and block the pores, producing an ultra filter which would impede or even stop filtration.

With the object of testing the correctness of these hypotheses, the following experiments were accordingly instituted.

Experiment I. Separate volumes of plasma, undiluted or diluted, were made 20, ... 54 per cent. of saturation with ammonium sulphate. The refractive indices of these mixtures were measured and the protein still remaining in solution after the addition of the various amounts of ammonium sulphate solution was calculated, the results, for the purposes of comparison, being expressed in terms of the original plasma.

The various mixtures were then heated to a temperature of 61° or 63° according to the directions given in Banzhaf's paper, and the protein contents of the filtrates from samples of the mixtures were measured at the end of the heating process. The results have been embodied in Tables II, III, IV, and V and in Figure 1.

(*a*) The curve plotted in Figure 1, giving the relation between the concentration of ammonium sulphate in the mixtures and the amount of protein remaining in solution, shows that precipitation occurs mainly at

percentages of saturation with ammonium sulphate greater than 28. However the gradient of the curve indicates no critical points for the precipitation of the eu- or pseudo-globulin fraction or of the serum albumins.

(*b*) After heating the mixtures containing concentrations of ammonium sulphate of less than 28 per cent. of saturation, clear liquids could not be obtained—the filtrates remained permanently opalescent and even on long standing the particles of the suspension did not coagulate sufficiently to be retained by filter paper.

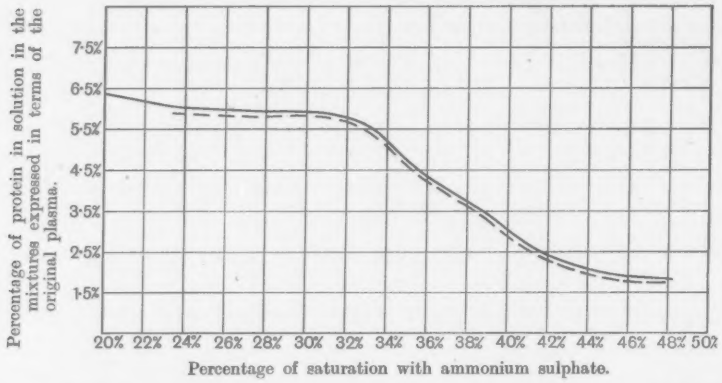
Percentage of protein in solution in the mixtures expressed in terms of the original plasma.
7·5%
6·5%
5·5%
4·5%
3·5%
2·5%
1·5%
20% 22% 24% 26% 28% 30% 32% 34% 36% 38% 40% 42% 44% 46% 48% 50%
Percentage of saturation with ammonium sulphate.

Fig. 1. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate during the process of heating the mixtures just to a temperature of 61° and keeping them at that temperature for a few minutes only.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ at room temperature.

The curve — — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ and heated just to 61°.

(*c*) If the mixtures were kept at a temperature of 61° or 63° for a few minutes only, as advocated by Banzhaf, there was, during the heating process, no appreciable change in the protein content of the solution indicated by the refractometer (Tables II and III).

(*d*) If the mixtures were kept at a temperature of 61° or 63° for three-quarters of an hour, or longer (Table IV), then there was, during the heating process, a considerable change in the amount of protein in solution.

(*e*) If *undiluted* plasma were made various percentages of saturation with ammonium sulphate and the mixtures heated to 63° and kept at that temperature for a few minutes only, there was no appreciable change in the protein content of the solutions during the heating process (Table V).

TABLE II.

Oxalated plasma was diluted with half its volume of water and made various percentages of saturation with ammonium sulphate. The mixtures were then heated to a temperature of 61° *and kept at that temperature for a few minutes, the heating process taking two hours in all.*

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma[1]		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	6·49		
20	6·30	6·10	3·2
24	5·91	5·80	1·9
26	5·89	5·79	1·6
28	5·84	5·61	4·0
30	5·85	5·76	1·6
31	5·53	5·36	3·1
32	5·18	5·12	1·1
33	5·18	5·04	2·7
34	4·78	4·69	1·9
36	4·25	4·12	3·1
38	3·84	3·74	2·6
40	2·80	2·86	−2·1
44	2·02	1·90	6
48	1·90	1·90	0

[1] Throughout these tables it has not been deemed necessary to make corrections for the fact that the addition of x cc. of ammonium sulphate solution to $100-x$ cc. of serum does not necessarily yield a volume of 100 cc. of mixture.

TABLE III.

Oxalated plasma was diluted with half its volume of water and made various percentages of saturation with ammonium sulphate. The mixtures were heated to a temperature of 63° *and kept at that temperature for a few minutes.*

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	8·36		
25	7·35	(Too opalescent to be read)	
28	7·33	6·98	5·0
29	6·81	6·52	4·3
30	6·08	5·92	2·7
32	5·92	5·61	5·3
34	5·48	5·44	0·74
35	5·24	5·11	2·5
40	4·83	4·68	3·1
46	3·39	3·31	2·4
48	2·08	2·14	−3
50	1·31	1·31	0

TABLE IV.

Oxalated plasma diluted with half its volume of water was made various percentages of saturation with ammonium sulphate. The mixtures were heated to 61° *or* 63° *and kept there for three-quarters of an hour.*

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma			Percentage conversion of *soluble* into *insoluble* protein during the heating process in periods	
	(*a*) Before heating	(*b*) After heating just to 61°	(*c*) After 0·75 hr further heating at 61° or 63°	(*b*)	(*c*)
Original plasma	6·49				
30	5·76	5·63	4·28[1]	2·3	25·7
33	5·19	5·02	3·78[2]	3·3	27·2
34	4·81	4·67	3·55[3]	3·1	26·1

[1] Heated to 61° for 1 hour. [2] Heated to 62° for 0·75 hour. [3] Heated to 63° for 0·75 hour.

TABLE V.

Undiluted oxalated plasma was made various percentages of saturation with ammonium sulphate. The mixtures were heated to a temperature of 63° *and kept at that temperature for a few minutes only.*

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	7·14		
28	6·23	5·97	4·2
30	5·98	5·83	2·4
31	5·73	5·43	5·3
32	5·33	4·98	6·6
33	5·17	4·90	5·3

Experiment II. That there was no appreciable change in the pseudo-globulin content of the mixtures during the process of heating was demonstrated gravimetrically as follows.

Several volumes of plasma, both diluted and undiluted, were taken and to them was added respectively ammonium sulphate solution in quantity to make a series of percentage solutions of varying concentration. Each series was duplicated. The same process was carried out with serum.

Of the duplicates of each mixture the one was dialysed without heating and the other previous to dialysis was heated to a temperature of 61° or 63°, and kept there for a few minutes only. After dialysis the residues were made up to a certain volume, aliquot parts of the clear liquid were taken and gravimetric estimations were made on the protein contained therein. Two protein estimations were made on each dialysis residue:

(*a*) The total protein present soluble in saturated brine;

(*b*) The total amount of protein present not precipitated by 50 per cent. saturated ammonium sulphate.

In some cases the total protein in solution in the dialysis residues was estimated gravimetrically and also by means of the refractometer. Gravimetric estimations were invariably slightly higher than those obtained from the refractometer readings, thus indicating that in the dialysis residues there is, in colloidal suspension, a certain amount of insoluble euglobulin, the particles of which are too small to be retained by filtration through paper.

The gravimetric estimations, collected in Table VI, confirm the evidence obtained from the use of the refractometer that there has been no appreciable conversion of pseudoglobulin into euglobulin during the heating process.

The evidence thus afforded in Experiments I and II indicates that Banzhaf's supposition that the heating process in his new method serves to convert some of the pseudoglobulin into euglobulin does not provide such an explanation of the changes taking place as can be verified by quantitative examination.

At first sight it is difficult to reconcile the conclusions thus to be drawn from a study of these data with the obvious fact that, during the heating process of the serum mixture the bulk of the precipitate visibly increases. The following experiment, however, serves to throw light on this apparent paradox.

Experiment III. Serum mixtures were made from 25 to 54 per cent. of saturation with ammonium sulphate and, after standing at room temperature in stoppered flasks for 2 or 3 hours, were filtered. The clear filtrate was divided into two portions and kept in stoppered flasks. In the one portion, which stood at room temperature for several days, the clear liquid, within a few hours, became cloudy, and there was then a deposition of solid protein which gradually increased in bulk. The other portion was heated to 63° (two and a half hours being taken just to reach that temperature) and in every case a precipitate was formed, although at dilutions below 28 per cent. of saturation with ammonium sulphate the precipitate was so

TABLE VI.

Gravimetric estimation of the protein "soluble in saturated brine" in solution in the serum mixtures before and after being subjected to the heating process.

Volume of plasma or serum previously diluted with half its volume of water	Percentage protein content of original plasma	Electrolytes added	Treatment before dialysis	Proportion of mixtures taken for dialysis	Total protein soluble in saturated brine solution		Protein not precipitated by making dialysis residue 50 % saturation with $(NH_4)_2SO_4$	
					(*a*) in the dialysis residue	(*b*) in 100 cc. of original plasma	(*a*) in the dialysis residue	(*b*) in 100 cc. of original plasma
					g.	g.	g.	g.
A. Plasma (oxalated)	7·14							
(i) 50 cc.	—	made 30 % of saturation with $(NH_4)_2SO_4$	unheated	whole = ppte + liquid	1·324	3·972	0·650	1·950
(ii) 50 cc.	—	,,	heated	whole	1·325	3·975	0·649	2·060
B. Plasma (oxalated)	7·14							
(i) 50 cc.	—	made 32 % of saturation with $(NH_4)_2SO_4$	unheated	,,	1·424	4·272	0·635	1·905
(ii) 50 cc.	—	,,	heated	,,	1·576	4·728	0·6365	1·910
C. Plasma (oxalated)	5·76							
(i) 50 cc.	—	,,	unheated	,,	0·995	2·984	0·602	1·806
(ii) 50 cc.	—	,,	heated	,,	1·015	3·045	0·602	1·805
D. Serum (carbolised)	5·51							
(i) 50 cc.	—	,,	unheated	,,	0·678	2·034	—	—
(ii) 50 cc.	—	,,	heated	,,	0·600	1·800	—	—

finely divided that the liquid could not be filtered clear for refractometer readings. In all cases, whether the filtrates had been heated or not, although there was the visible formation of solid matter from the previously clear filtrate, there was no change in the refractive indices of the solutions, thus indicating no further change in the protein content of the solution after the immediate effect due to the addition of the electrolyte at room temperature. Moreover, this delayed, slowly forming, precipitate was not a special feature of the mixtures containing a percentage of saturation with ammonium sulphate insufficient to precipitate completely the euglobulin from solution, *i.e.* up to 33 per cent. of saturation with ammonium sulphate, for it occurred in all of the ammonium sulphate-serum mixtures.

We are therefore led to no other conclusion than that the effect of heating the serum mixtures just to 61° or 63° merely serves to accelerate a change which takes place slowly at ordinary room temperature, and that whatever this change may be, it is not concerned with the conversion of the pseudoglobulin into the euglobulin.

Light may however be thrown on the question by a consideration of the following observations. Proteins belong to the hydrophile type of colloids, and in solution form an emulsoid system. It has been demonstrated by Chick and Martin [1913] and others that if a high concentration of electrolytes be added to a solution of a protein, there is withdrawal of water from the internal phase of the emulsoid system, and if the withdrawal be sufficient, this phase becomes rigid and is eventually precipitated. In the salting out of proteins the emulsoid is thus first converted into a suspensoid system. Applying these observations to the salting out of serum proteins, it is probable that in the fractional precipitation, with additions of ammonium sulphate to less than 33 per cent. of saturation, the euglobulin is first changed from the emulsoid to the suspensoid type of colloid: the suspensoid is in part converted into a suspension and the latter settles as a precipitate. The protein thus converted into a suspensoid or suspension will no longer have a marked effect on the viscosity or on the refractive index of the solution—even though the particles be neither visible to the naked eye, nor retained by filter paper.

The experimental evidence given above (Experiment III) shows that the complete agglutination of the particles of the suspensoid to those of a perceptible precipitate takes place slowly at ordinary temperatures, and that the effect of heating the liquids in the new process merely serves to increase the velocity of this change. Clearly then, the change thus taking place is not that suggested by Banzhaf, viz. the conversion of pseudoglobulin

into euglobulin, but rather a more simple physical process involving the agglutination of the particles of euglobulin already thrown out of emulsoid suspension by the ammonium sulphate. This agglutination permits of easy separation of the euglobulin by filtration through paper.

If, for some reason or other, in the routine concentrations of batches of sera or plasma, the precipitated euglobulin is not completely agglutinated during the heating process, then difficulties will be encountered in

TABLE VII.

Experiment to demonstrate the power of insufficiently agglutinated particles in a badly filtering heated serum mixture to convert an ordinary filter paper into an ultra filter.

Oxalated plasma, diluted with half its volume of water and made various percentages of saturation with ammonium sulphate had been heated just to 61°. The serum mixtures filtered badly; at first there was an opalescent filtrate but the filtration soon slackened and almost ceased.

The protein content of the first opalescent filtrate was estimated and compared with that of the slowly forming clear drops of filtrate collected a short time before the filtration ceased.

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma			The self made ultra filter has effected the removal of soluble protein to the extent of
	(*a*) Before heating	(*b*) After heating		
		(i) First filtrate slightly opalescent	(ii) Clear drops just before filtration ceased	
Original plasma	6·49			
24	5·91	5·80	4·68	20·8 %
26	5·89	5·80	4·69	20·3 %
28	5·84	5·61	4·86	16·8 %
30	5·85	5·76	5·30	9·5 %
31	5·53	5·36	5·22	5·6 %

the subsequent process of filtration. Some of the insufficiently agglutinated aggregates readily pass through the pores of the filter paper, while others, not sufficiently large to be retained on the surface of the paper, either partially or wholly block the pores of the paper and so retard the process of filtration. (Table VII shows that the partially blocked paper may act as an ultra filter.) The euglobulin thus passing into the filtrate will be precipitated with the pseudoglobulin and antitoxin at one-half saturation with ammonium sulphate. Theoretically speaking, when the precipitated pseudoglobulin and antitoxin

containing a small amount of euglobulin is dialysed until the salt content approaches that of tap water, the pseudoglobulin and antitoxin only should be in solution, but it is generally accepted that the presence of one protein in solution protects another from precipitation.

We have seen (Experiment II) that even in dilute dialysis residues a small amount of euglobulin remained in colloidal suspension. Since, in routine concentrations, the dialysis residues may contain up to and even more than 20 per cent. of pseudoglobulin, the protecting power of the latter in respect of the euglobulin is enhanced. The euglobulin thus in colloidal suspension will greatly retard, if not render impossible, the filtration of the final product through filter candles.

The impediment to filtration in the new process caused by the suspensoid particles of euglobulin was in practice removed by allowing the dialysis residue to stand for a prolonged period. This tedious method has had to be resorted to frequently.

II. *An investigation of the rôle of Sodium Chloride in improving the Banzhaf new One Fraction Method.*

Having ascertained the value of the heating process in the new method, the question then arose as to the part played by the sodium chloride in improving the concentration process. Did the addition of salt to the serum mixtures serve to induce the desired conversion of pseudoglobulin into euglobulin or did it merely exert some stimulating effect on the agglutination of the aggregates of the euglobulin, a process which, from the experimental work in Part I, seems to take place slowly?

In order to elucidate this point, routine concentrations in which 1·5 per cent. of solid sodium chloride had been added to serum or plasma mixtures which had been made 30 per cent. or 31 per cent. of saturation with ammonium sulphate were carefully examined at different stages of the heating process. As before, samples were taken from the mixtures and filtered, and the protein content of the clear filtrates was estimated from the difference between the refractometer readings of the clear filtrate before and after coagulation of the protein therein. For purposes of comparison the protein content of the mixtures at various stages in the heating process was expressed in terms of the original serum or plasma.

Up to the present the concentration of 20 batches each of not less than 50 litres of plasma or serum has been followed; the data with regard to

three typical concentrations are given in Table VIII. In no case has any difficulty been experienced in the filtration of the hot liquids or of the final product so far as this latter has up to the present been filtered. In no case has there been any appreciable change in the protein content of the mixtures during the heating process.

From these observations we are led to the conclusion that the effect of the heating process is to agglutinate the particles of euglobulin already converted from the emulsoid to the suspensoid state. This process, which seems to be incomplete in some serum mixtures, is stimulated by the addition of sodium chloride to the same mixtures.

TABLE VIII.

Changes taking place during the heating process to 63° *or* 65° *in the protein content of routine concentration serum mixtures which have been made* 30 *or* 31 *per cent. of saturation with ammonium sulphate and* 1·5 *per cent. with solid sodium chloride.*

Amount of oxalated plasma concentrated	Protein content of original plasma	Percentage of saturation with ammonium sulphate	Duration of time of heating of mixtures	Temperature of mixtures at which samples were taken	Residual percentage of protein in solution in the filtrates of the serum mixtures, calculated from the refractometer readings and expressed in terms of the original plasma
Diphtheria	7·00	31	0	Room. temp.	5·11
50 litres			2 hrs	57°	5·00
			2 hrs 35′	61°	5·02
			3 hrs	63°	4·93
Tetanus	7·47	30	1 hr 55′	57°	6·82
50 litres			2 hrs 25′	61°	6·60
			2 hrs 55′	63°	6·60
Tetanus	6·73	30	0	Room temp. = 16°	5·19
50 litres			1 hr 35′	57°	5·28
			2 hrs 15′	61°	5·28
			2 hrs 35′	65°	5·12

Additional evidence on this point was furnished by the following experiments.

Experiment IV. Serum or plasma (diluted or undiluted) was made from 25–50 per cent. of saturation with ammonium sulphate and solid sodium chloride was added to the mixture to the extent of 1·5 per cent. The protein content of the filtrates of samples of the mixtures was estimated from the refractometer readings as in the previous experiments. The mixtures were then subjected to various treatments.

(*a*) The mixtures were heated to 63° and kept at that temperature for a few minutes, the process in all taking from 2 to 2·5 hours. (Table IX.)

It will be seen from the measurements of the refractive indices of the solutions and from the curves in Figure 2 that there was no appreciable change in the pseudoglobulin content of the mixtures during the process of heating.

(*b*) The mixtures were heated to a temperature of 67° and kept at that temperature for 10 minutes. (Table X and Figure 3.)

TABLE IX.

Oxalated plasma diluted with half its volume of water, made various percentages of saturation with ammonium sulphate and 1·5 *per cent. solid sodium chloride added to the mixtures. The mixtures were heated to a temperature of* 63° *and kept at that temperature for a few minutes.*

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates from the mixtures and expressed, for purposes of comparison, in terms of the original undiluted plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	8·30		
29	6·93	6·66	4·0
30	6·73	6·52	3·1
31	6·38	6·14	3·8
32	5·97	5·89	1·36
33	5·09	4·85	0·8
34	4·81	4·70	2·96
36	3·73	3·64	2·5
39	2·44	2·39	2·1
40	2·19	2·19	0
46	2·15	2·15	0
54	0·574	0·582	−1·6
Original plasma	7·14		
29	5·68	5·44	4·3
30	5·30	5·25	1
31	5·12	5·08	0·8

From the results it is clear that heating the serum mixtures to this higher temperature is accompanied by a considerable change in the protein content of the solutions.

(*c*) The mixtures were heated to a temperature of 63° and kept at that temperature for 1·25 hours. (Table XI and Figure 3.)

Obviously the prolongation of the time of heating at 63° also gives rise to considerable change in the protein content of the solution.

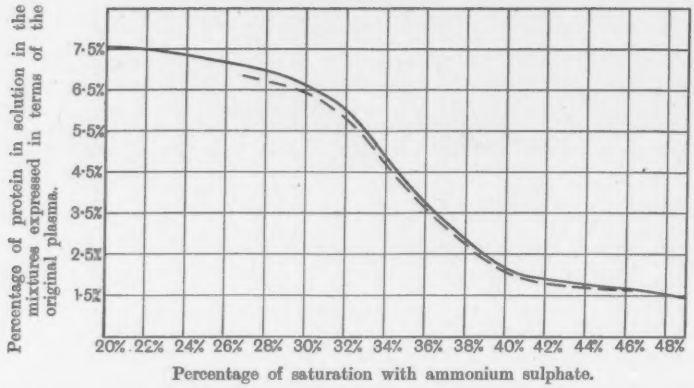
Percentage of protein in solution in the mixtures expressed in terms of the original plasma.
7·5%
6·5%
5·5%
4·5%
3·5%
2·5%
1·5%
20% 22% 24% 26% 28% 30% 32% 34% 36% 38% 40% 42% 44% 46% 48%
Percentage of saturation with ammonium sulphate.

Fig. 2. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate and 1·5 per cent. with solid sodium chloride during the process of heating the mixtures just to a temperature of 63° and keeping them at that temperature for a few minutes only.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ at room temperature.

The curve — — — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ heated to 63° and kept at that temperature for a few minutes only.

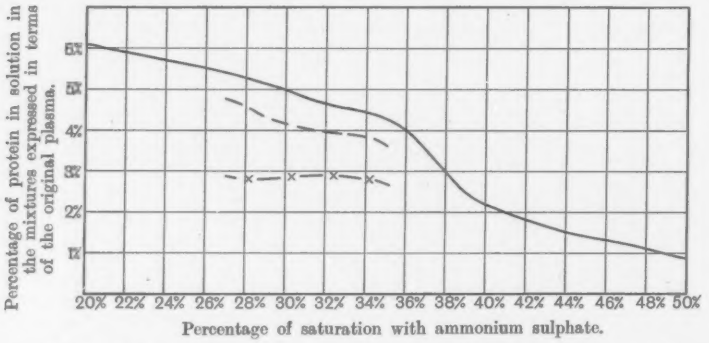
Percentage of protein in solution in the mixtures expressed in terms of the original plasma.
6%
5%
4%
3%
2%
1%
20% 22% 24% 26% 28% 30% 32% 34% 36% 38% 40% 42% 44% 46% 48% 50%
Percentage of saturation with ammonium sulphate.

Fig. 3. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate, during the process of heating (*a*) just to a temperature of 67° and (*b*) to a temperature of 63° and keeping at that temperature for 1¼ hours.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ at room temperature.

The curve — — — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ and heated just to 67°.

The curve — × — × — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ and heated to 63° and kept at that temperature for 1¼ hours.

Experiment V. That there was no appreciable change in the pseudoglobulin content of the mixtures during the heating process adopted in the new method was also demonstrated gravimetrically.

Several volumes of serum or plasma, diluted or undiluted, were respectively made various percentages of saturation with ammonium sulphate, and 1·5 per cent. of solid sodium chloride was then added. Each series was duplicated and of the duplicates the one was dialysed without heating and the other, previous to dialysis, was heated to a temperature of 63° and kept at that temperature for a few minutes only—the heating process taking 2 to 2·5 hours in all.

TABLE X.

Oxalated plasma diluted with half its volume of water was made various percentages of saturation with ammonium sulphate and 1·5 *per cent. of solid sodium chloride was added to the mixtures which were then heated to a temperature of* 67°.

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates from the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	6·23		
28	5·34	4·66	12·9
29	5·14	4·24	17·7
30	4·98	4·00	20·0
31	4·97	4·25	14·5
32	4·62	3·99	13·6
33	4·54	4·05	11·0
34	4·56	3·96	10·4

The protein estimations were made on each dialysis residue, viz.:

(*a*) The total protein present soluble in saturated brine solution.

(*b*) The total amount of protein not precipitated by 50 per cent. of saturation with ammonium sulphate.

The results showed that, during the heating process, there was no appreciable conversion of pseudoglobulin into euglobulin.

From the results given in Tables VI and XII it is interesting to note that the amount of protein precipitated from the dialysis residues by saturation with brine depends upon the original dilution of the serum or plasma. This is a generally accepted fact (Banzhaf-Gibson).

In certain cases the whole serum mixture, *i.e.* precipitate and liquid, as well as controls of the filtrate alone were dialysed, when it was observed that the pseudoglobulin was much greater in amount in the former than in the latter case: that is to say that the precipitate brought down by the ammonium sulphate was in reality a mixture of eu- and pseudo-globulin.

Further, in those instances in the above experiment in which oxalated plasma was used, the dialysis residues of the unheated plasma mixtures contained the insoluble fibrin and euglobulin formed into a jelly-like clot. In the dialysis residues of the heated mixtures these insoluble proteins were present as a flocculent and readily subsiding precipitate.

Experiment VI. Further experimental work was then undertaken to ascertain the effect of sodium chloride on the solubility of serum proteins under different conditions.

TABLE XI.

Oxalated plasma, diluted with half its volume of water, was made various percentages of saturation with ammonium sulphate and 1·5 *per cent. solid sodium chloride added to the mixtures which were then heated to* 63° *and kept at that temperature for* 1·25 *hours.*

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates from the mixtures, and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	6·23		
28	5·44	2·81	48·6
30	5·29	2·89	45·5
32	4·56	2·80	39·0
34	4·62	2·86	38·4

(*a*) *The effect of heating a saturated brine solution of pseudoglobulin.*

The globulin precipitate thrown down by making plasma 50 per cent. of saturation with ammonium sulphate was suspended in saturated brine solution and after standing at room temperature for 48 hours was gradually heated to a temperature of 61°, the process taking 2 hours in all.

From the use of the refractometer it was ascertained that at room temperature (after 48 hours) the saturated brine contained protein in solution corresponding with a value of 6·3 per cent. in the original plasma. After

TABLE XII.

Gravimetric estimation of the protein "soluble in saturated brine" present in serum mixtures made various percentages of saturation with ammonium sulphate and 1·5 per cent. with sodium chloride, before and after being heated to 63° and kept at that temperature for a few minutes only.

	Percentage protein content of citrated plasma taken	Dilution of plasma	Volume of diluted plasma taken	Percentage of saturation with $(NH_4)_2SO_4$	Treatment of mixtures *previous to dialysis*	Total protein soluble in saturated brine		Protein not precipitated at 50 per cent. of saturation with $(NH_4)_2SO_4$	
						(*a*) the dialysis residue	(*b*) 100 cc. of original serum or plasma	(*a*) in the dialysis residue	(*b*) in 100 cc. of original plasma or serum
A.	7·14	undiluted	100 cc.	29	The mixture, after standing 2 hrs, was filtered. Of the clear filtrate				
					(i) 50 cc. were unheated	1·074	3·024	0·633	1·880
					(ii) 50 cc. were heated	1·098	3·091	0·620	1·745
B.	7·14	undiluted	100 cc.	31	The mixture, after standing 2 hrs, was filtered and of the clear filtrate				
					(i) 50 cc. were unheated	1·596	4·62	0·624	1·800
					(ii) 50 cc. were heated	1·700	4·92	0·600	1·714
C.	7·14	undiluted	2 lots of 50 cc. each	31	The whole mixtures, *i.e.* ppte and liquid, were respectively taken and				
					(i) unheated	2·68	5·36	1·17	2·34
					(ii) heated	2·56	5·12	1·13	2·26
D.	5·76	diluted with ½ its vol. of H_2O	100 cc.	31	The mixture, after standing 2 hrs, was filtered and of the clear filtrate				
					(i) 50 cc. were unheated	0·234	0·641	—	—
					(ii) 50 cc. were heated	0·227	0·621	—	—

raising the temperature of the brine solution just to 61°, the protein in solution had decreased to the value of 2·48 per cent.

During the heating process there had thus been a conversion of 60·6 per cent. of the soluble into insoluble protein; at the same time there was considerable loss of antitoxic units from the solution.

TABLE XIII.

The effect of the prolonged heating of plasma, diluted with one-third its volume of water and containing 1·5 *per cent. of sodium chloride, upon* (*a*) *the solubility of the serum proteins and* (*b*) *the precipitating power of* 30 *per cent. of saturation with ammonium sulphate.*

Length of time diluted plasma or serum was heated	Temperature of heated liquids	Residual percentage of protein in solution: (*a*) in the diluted plasma or serum at various stages in the heating process calculated from the refractometer readings of the heated liquids and expressed, for purposes of comparison, in terms of the original plasma	Residual percentage of protein in solution: (*b*) after making the heated liquids 30 per cent. of saturation with $(NH_4)_2SO_4$, calculated as in (*a*)	Percentage increase in the amount of soluble protein precipitated by 30 per cent. of saturation with $(NH_4)_2SO_4$
Plasma (*oxalated*)				
0 hrs	Room temperature	7·38	5·12	—
3 hrs	57°	7·05		
7 hrs	57°	7·01	4·84	5·5
Serum (*carbolised*)				
(1) 0 hrs	Room temperature	6·47	5·82	
15 hrs	57°	5·99	3·81	34·6
(2) 0 hrs	Room temperature	6·95	5·41	
15 hrs	57°	5·77	3·13	42·2

(*b*) *Prolonged heating of serum or plasma to which* 1·5 *per cent. sodium chloride had been added.*

Plasma and serum were each diluted with one-third of their volume of water, 1·5 per cent. of salt added, and heated to a temperature of 57° for periods varying from 6 to 15 hours.

The refractometer readings showed that during the heating process there was a slight change in the protein content of the solutions, but this change did not take place with less than three hours' heating. Samples of the heated mixtures, taken after 6 and 15 hours' heating respectively, were made 30 per cent. of saturation with ammonium sulphate and the protein thrown out of solution compared with that precipitated by the same

saturation with ammonium sulphate in the original unheated serum or plasma. (Table XIII.)

The results show that the amount of protein remaining in solution after making the serum 30 per cent. of saturation with ammonium sulphate is practically the same for the unheated serum as for the serum heated to 57° for 6 hours. After 15 hours' heating there is a 30–40 per cent. increase in the precipitation of protein.

Further heating of the respective ammonium sulphate mixtures to a temperature of 61° was not accompanied by any increased change in the protein content of the solutions.

(*c*) *The heating of serum or plasma without the addition of salt.*

In order to ascertain whether the change thus taking place in (*b*) during the prolonged heating of plasma or serum with 1·5 per cent. sodium chloride was due to the specific action of the sodium chloride on the proteins of horse serum or was an inherent property of the proteins themselves, plasma and serum diluted with water were heated, *without the addition of salt*, in order to ascertain whether changes had been induced during the heating.

(*α*) *Changes in the solubility of the serum proteins.*

During the heating process there was slight deposition of proteins and samples of the heated liquids filtered with difficulty.

From Table XIV it will be seen that whether the serum or plasma had been heated at 63° for a short time or kept at lower temperatures for longer periods there was a slight change in the solubility of the proteins.

(*β*) *Changes in the precipitating power of* 30 *per cent. of saturation with ammonium sulphate.*

The results depicted in Table XV show that considerable changes may take place in the precipitating power of this reagent.

It has been previously demonstrated that the heating of the serum to 63°, when the liquid was kept at that temperature for a few minutes only, was not followed by any appreciable change in the amount of protein precipitated by 30 per cent. of saturation with ammonium sulphate. But if the liquids were kept at this temperature for 1 hour, or if the temperature were raised to 67°, then considerably more protein was precipitated (from 10 to 20 per cent. increase).

TABLE XIV.

The effect of the heating of serum or plasma on the solubility of the serum proteins [*No addition of* NaCl.]

Dilution of the oxalated plasma or serum	Temperature to which heated	Duration of the time of heating at the specified temperature	Percentage of protein in solution in the plasma—measured by means of the refractometer (*a*) Before heating	(*b*) After heating	Percentage conversion of *soluble* into *insoluble* protein during the heating process
(1) *Plasma* with ½ its vol. of H_2O	63°	10 min.	7·69	7·43	3·4
(2) *Plasma* with ½ its vol. of water	57°	0 hrs	5·85	—	
	57°	15 hrs	5·85	5·30	9·4
(3) *Plasma* with ½ its vol. of water	57°	0 hrs	6·14	—	
	57°	4 hrs	,,	5·99	2·4
	57°	15 hrs	,,	5·79	5·7
(4) *Carbolised serum*[1] with ½ its vol. of water	57°	0 hrs	5·59	—	
	57°	15 hrs	,,	3·79	32·2

[1] A considerable change always takes place during the heating of carbolised serum. This point is being investigated.

TABLE XV.

The effect of the prolonged heating of plasma and of serum respectively diluted with one third their volume of water upon the precipitating power of 30 *per cent. of saturation with ammonium sulphate.* [*No addition of* NaCl.]

Temperature reached	Duration of the heating at the specified temperature	Percentage of protein in solution at the stage in the heating process. Expressed in terms of the original plasma	Percentage of protein remaining in solution after making the liquid 30 per cent. of saturation with ammonium sulphate. Expressed in terms of the original plasma	Percentage change in the amount of dissolved protein precipitated by 30 per cent. of saturation with $(NH_4)_2SO_4$
Carbolised serum				
57°	0 hrs	5·59	4·53	
	15 hrs	3·79	2·42	46·0
Plasma				
57°	0 hrs	5·85	5·80	
	15 hrs	5·30	3·52	39·3
Plasma				
57°	0 hrs	6·14	5·78	
	4 hrs	5·99	5·48	5·2
	15 hrs	5·79	3·52	39·0
Plasma				
55°	0 hrs	7·39	5·35	
	15 hrs	6·89	4·81	10·0

If the plasma or serum were kept at a temperature of 55° for 15 hours there was a 10 per cent. increase in the precipitating power of 30 per cent. saturation with ammonium sulphate.

When the plasma or serum had been kept at 57° for 4 hours there was very little difference between the amounts of protein precipitated from the heated and unheated liquids by the addition of saturated ammonium sulphate to the extent of 30 per cent. of saturation.

After 15 hours' heating at 57° it was found that, on the addition of ammonium sulphate to 30 per cent. of saturation, from 39–47 per cent. more of the soluble protein was precipitated from the heated than from the unheated liquids.

It is thus obvious that the change taking place in the prolonged heating of serum or plasma with 1·5 per cent. sodium chloride in (*b*) is in no way dependent on the presence of the sodium chloride, as the same phenomenon takes place in the prolonged heating of serum or plasma to which no salt has been added.

That changes take place in the prolonged heating of solutions of pseudoglobulin has been shown by Banzhaf [1908] but from the details given in his paper it is difficult to understand the exact procedure adopted by him. Banzhaf suggests that the change is due to the conversion of pseudoglobulin into euglobulin, but from consideration of the work of Hardy [1899], Chick and Martin [1912], and Chick [1914] it is probable that the change taking place in (*b*) and (*c*) above is of the nature of a denaturation of proteins. For, evidently, during the prolonged heating of the serum or plasma, while the actual solubility of the proteins has been little changed their physical properties have become so altered that their precipitability by electrolytes is considerably increased.

In view of these observations it is at first sight not easy to picture the part played by the sodium chloride in rendering the concentration of antitoxic sera by Banzhaf's new process more certain of success. If it were merely a question of the better salting out of the euglobulin by increasing the dehydrating action exerted by the salt, then why should the addition of 1·5 per cent. sodium chloride to the 29 or 30 per cent. of saturation with ammonium sulphate serum mixtures yield a better and more easily filterable end product than that obtained from 31, 32, 33 per cent. or even 34 per cent. of saturation in the absence of sodium chloride.

Now Banzhaf and Gibson [1907] have stated that the salting out of euglobulin is influenced by the dilution of the plasma and they have

given what is, in their opinion, the optimum dilution for routine concentrations.

But, that factors other than the dehydrating effect of the salts added to the serum and the dilution of the serum proteins have to be considered, is shown by an examination of the data collected during the progress of this research. (Table XVI.)

TABLE XVI.

The percentage of protein remaining in solution in serum mixtures made 30 *or* 31 *per cent. of saturation with ammonium sulphate.*

Percentage protein content of the original plasma taken	Treatment of the diluted plasma		Residual percentage of protein remaining in solution in the mixtures and expressed in terms of the original plasma
	(*a*) Percentage of saturation with $(NH_4)_2SO_4$	(*b*) Percentage of NaCl in the mixtures	
7·14	30	—	4·93
6·47	,,	—	5·85
6·47	31	—	5·53
8·10	,,	—	6·29
8·36	,,	—	6·39
7·14	,,	—	5·74
6·71	30	1·5	5·16
6·73	,,	,,	4·19
7·38	,,	,,	4·97
7·14	,,	,,	5·20
7·38	,,	,,	5·12
6·38	,,	,,	5·81
5·51	,,	,,	4·53
5·76	,,	,,	5·80
6·04	,,	,,	5·76
6·23	,,	,,	5·29
8·32	,,	,,	6·39
8·32	31	1·5	5·97
7·14	,,	,,	5·12
7·28	,,	,,	5·11
7·00	,,	,,	5·11
6·71	,,	,,	4·61
7·47	,,	,,	6·80
7·28	,,	,,	4·42
6·79	,,	,,	5·59

It will be seen, that though the ammonium sulphate concentration and the protein concentration of some of the samples were practically identical, yet the amount of protein left in solution was considerably different. Evidently factors operated which had not been controlled.

It is believed that colloidal aggregates are kept in solution by their mutual

repulsion which is due to the electric charge they carry. If this charge be sufficiently reduced, then the forces keeping the particles apart diminish; at the iso-electric point the colloids will be most completely precipitated. In other words, the more nearly the hydrogen-ion concentration of the system approaches that of the iso-electric point for a particular protein, the more complete will be the precipitation of that protein.

Therefore, when dealing with the concentration of antitoxic sera on a large scale, we ought not to lose sight of the fact that the reaction of the serum mixtures is probably of more vital importance in the salting out process than has hitherto been recognised in the methods of practical value advocated for the concentration of antitoxic sera. The more nearly the reaction of the serum mixtures approaches the hydrogen-ion concentration at the iso-electric point of euglobulin, not only should there be more rapid and effective filtration, but more constant results should be obtained with regard to the percentage removal of protein from the solution.

In the Banzhaf new One Fraction Method no cognisance is taken of the reaction of the mixture and this may well be the secret of its uncertainty.

The alkalinity of blood is supposed to be due to the partial hydrolysis of certain sodium salts of weak acids, and if this be the case the introduction of a salt like sodium chloride should slightly reduce the alkalinity. It therefore follows that the stimulating effect of the sodium chloride may be due to the fact that it tends to make the hydrogen-ion concentration of the mixtures approach more nearly that required for the optimum precipitation of euglobulin.

In support of this theory it may be mentioned that during the past few months I have had occasion to study the heat coagulation of 350 samples of serum. With some samples the heat coagulation could be readily brought about. With others the formation of a readily filterable coagulum could only be induced after the addition of from 1 to 6 per cent. of sodium chloride, or by the addition of corresponding amounts of a standard solution of acetic acid. The sodium chloride and the acetic acid were interchangeable and apparently had the same effect whether used separately or whether a certain proportion of the one was replaced by the corresponding proportion of the other.

In this connection experiments are being conducted with the object of ascertaining the influence of alteration of the reaction (hydrogen-ion concentration) of the serum mixtures on the precipitation and filtration of the eu- and pseudo-globulin and antitoxin fractions. For, although the

desired improvement in filtration may be obtained by the addition of acetic acid to the more alkaline sera we must not lose sight of the fact that, as salt is known to exert a protecting action on antitoxin, it may be better to adjust the reaction of the medium by means of salt rather than by the addition of acid. The results will be dealt with in a future communication.

Summary.

1. The advantages claimed by Banzhaf for his new One Fraction process are sometimes vitiated by the fact that a final product is obtained which, owing to the presence of an opalescent suspension presumably of euglobulin, cannot be filtered except after long standing.

2. The successful preparation of an easily filterable end product depends on the initial heating of the serum mixtures.

3. The heating process advocated in the new method does not cause a conversion of pseudoglobulin into euglobulin but merely serves to complete the aggregation of the euglobulin into particles sufficiently large to admit of separation by filtration.

4. The uncertainty with regard to the successful heating of the serum mixtures can be obviated by a slight modification of the described technique, viz. by the addition of 1·5 per cent. sodium chloride to the serum mixtures.

5. The following observations have been made with regard to the effect of the addition of sodium chloride to the serum mixtures:

(*a*) During the process of heating the mixtures to 63° or 65° the addition of salt does not induce changes in the amount of proteins still remaining in solution, but if the temperature be raised to 67° or if the mixtures be kept for one hour or more at 63°, then a considerable change in the protein content of the solution takes place.

(*b*) If a solution of pseudoglobulin and antitoxin in saturated brine be heated to a temperature of 61° and kept at that temperature for a few minutes only, there is a conversion of 60 per cent. of soluble into insoluble globulin, but at the same time there is loss of antitoxic units.

(*c*) Prolonged heating of serum or plasma diluted with water and containing 1·5 per cent. sodium chloride leads to an increase in the precipitating power of 30 per cent. of saturation with ammonium sulphate. This change, however, is not due to the presence of the sodium chloride. The prolonged heating of diluted plasma or serum gives rise to the same phenomenon.

Banzhaf considers that the change thus produced in the serum is that of pseudoglobulin into euglobulin, but it seems more probable that the prolonged heating serves to denaturate the protein in solution and to make it more readily precipitable by saturation with sodium chloride or by 30 per cent. saturation with ammonium sulphate.

6. It is possible that the addition of sodium chloride exerts a favourable influence on the filtration processes by virtue of an adjustment of the hydrogen-ion concentration of the serum mixtures.

REFERENCES.

Banzhaf (1908). *Proc. Soc. Exp. Biol. Med.*, **5**, 8; *Collected Studies, Research Lab. Dept. Health*, New York, 1908–9, **4**, 225.

—— (1909). *Collected Studies, Research Lab. Dept. Health*, New York, **4**, 230.

—— (1913). *Collected Studies, Research Lab. Dept. Health*, New York, **7**, 114.

Banzhaf and Gibson (1907). *Collected Studies, Research Lab. Dept. Health*, New York, **3**, 97.

Chick (1914). *J. Pathol. Bacteriol.*, **19**, 131.

Chick and Martin (1912). *J. Physiol.*, **45**, 251.

—— —— (1913). *Biochemical J.*, **7**, 380.

Hardy (1899). *J. Physiol.*, **24**, 158.

Reiss, Emil (1904). *Verh. 76 Versammlung deutsch. Naturforscher und Ärzte*, Breslau, 36.

Robertson, Brailsford (1915). *J. Biol. Chem.*, **22**, 233.

XXV. THE ESTIMATION OF CALCIUM.

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(*Received May 24th, 1916.*)

Some time ago Professor Andrewes [1915] was engaged in estimating the calcium in the aortas of normal and diseased persons. For that purpose the aortas were calcined until all organic matter was destroyed. After this operation the residue was worked up for the estimation of calcium by a slight modification of Aron's method [1907]. Certain aortas yielded a residue which only dissolved in strong sulphuric acid after many hours' heating. It was to overcome difficulties of this nature that the following method was devised. We found that a calcined residue which only dissolved in sulphuric acid after very prolonged heating would dissolve completely and in a few moments if heated with a solution of phosphoric acid of suitable strength. The calcium in such a solution can be estimated by the usual volumetric and gravimetric methods, which have for their basis the precipitation of calcium oxalate.

The Method.

Of a normal organ such as heart, or muscle, 100 grams of the substance are required; of an aorta rich in calcium very much less will suffice; of urine 100 cc. is a suitable quantity. The organ or tissue is dried at 100° and incinerated in the usual way; the urine is evaporated at first on the water bath, then dried at 120° and incinerated as before. To the residue a solution of phosphoric acid is added: if much calcium is present, 20 cc. of an acid of sp. gr. 1·2 (1 vol. syrupy phosphoric acid to 3 vols. water): if little, 10 cc. or even less. On warming, the calcium dissolves completely. This solution can be used direct for a volumetric determination, but it must be filtered and the residue washed for a gravimetric determination. The liquid is diluted

with 50 or 100 cc. of water according to the amount of calcium present and then a solution of oxalic acid containing 37·8 g. $C_2H_2O_4 2H_2O$ per litre is added so as to afford about five times the theoretical amount of oxalic acid. Thus in practice if the amount of calcium is about 0·2 g. reckoned as calcium carbonate, 100 cc. of the oxalic acid would be added. Precipitation is rapid, and the precipitate is crystalline, but a little calcium remains in solution. To effect complete precipitation a volume of ammonia equal to the volume of the oxalic acid used is then added, the strength of the ammonia being exactly equivalent to that of the oxalic acid, namely 10·2 g. of NH_3 per litre. If magnesium is present the ammonia must be added slowly and with shaking from a burette. The precipitated solution is allowed to stand for one hour: it will then filter quickly and perfectly clear. If ammonium oxalate be used, instead of oxalic acid followed by ammonia as above, the precipitate is far less crystalline and filters very badly.

A. *Volumetric Determination.*

In this method we make use of a small conical Buchner funnel so as to minimise the amount of washing needed to remove ammonium oxalate from the filter paper. Seven washings with cold distilled water are sufficient for this. The precipitate and paper are transferred to the same beaker in which the precipitation was effected; 10 cc. of sulphuric acid (1 vol. water : 1 vol. pure concentrated acid) are added; the solution heated nearly to boiling and titrated with decinormal permanganate. The sulphuric acid sometimes consumes a few drops of permanganate so that a blank experiment should be made.

B. *Gravimetric Determination.*

This is done in the usual way and needs no description.

Results.

As a check on the method we determined the percentage purity of a sample of commercial calcium carbonate sold as "Calcium Carbonate Reagent."

4·84 g. were dissolved in 25 cc. of syrupy phosphoric acid and made up to 250 cc. with water: 10 cc. were taken, and 50 cc. of water, 100 cc. of oxalic acid and 100 cc. of ammonia were added in every case.

Volumetric Results.

N/10 permanganate required	Percentage $CaCO_3$ found
39·8 cc.	—
39·7 „	102·6
39·8 „	—

Gravimetric Results.

Weight of CaO obtained	Percentage $CaCO_3$ found
0·1104	101·8
0·1104	101·8
0·1102	101·5

By solution of the carbonate in excess of hydrochloric acid and back titration by standard caustic soda we obtained in three experiments 100·64, 101·08, 101·25.

The sample evidently contained more calcium than corresponds to the formula $CaCO_3$: we found in fact that it contained some calcium hydroxide. By solution of some of the carbonate in hydrochloric acid and precipitation with ammonium carbonate, we obtained a specimen of really pure calcium carbonate which gave us a percentage of 100·14.

We now give examples of the determination of calcium in various substances by our own method and those of Aron [1907] and McCrudden [1910, 1911].

Urine. 100 cc. used in each experiment.

Percentage CaO.

Our method	Aron's method	McCrudden's method
0·072	0·071	0·074
0·045	0·044	0·045
0·033	0·032	0·032
0·041	0·033	0·040

Aorta. Portions of the same aorta were used for the determinations by us and by Professor Andrewes working independently.

Percentage CaO in dried Aorta.

	Our method	Andrewes by Aron's method
I	1·31	1·29
II	8·06	7·45
III	1·91	1·72

Calcium in the presence of Magnesium.

To test the validity of the method where calcium is accompanied by magnesium we determined these two elements in solutions, which contained a constant amount of calcium as oxide, and half, once and twice as much magnesium as oxide. The calcium was determined as described and the filtrate and washings from the calcium oxalate were concentrated to about 300 cc., 30 cc. of concentrated ammonia added and the mixture allowed to stand over night. The ammonium magnesium phosphate was then filtered off, washed, dried, ignited and weighed. Our first experiments were made with mixtures of calcium and magnesium phosphate and it appeared as if the magnesium were partly precipitated with the calcium oxalate for, as the proportion of magnesium rose from nought to twice the calcium, the amount of calcium increased. On testing the magnesium phosphate it was found to contain calcium. The use of phosphates was therefore abandoned and solutions of calcium and magnesium oxides in phosphoric acid were used. Of the calcium solution 10 cc. were used and 50 cc. of water added; but when calcium and magnesium were present together, 10 cc. of calcium solution and 150 cc. of water were used with 10, 20, and 30 cc. of the magnesium solution.

Results.

cc. CaO	cc. MgO	CaO found		MgO found
		Volumetric	Gravimetric	
10	0	0·1014	—	—
10	0	0·1014	—	—
10	0	—	0·1018	—
10	0	—	0·1015	—
0	10	—	—	0·0537
10	10	0·1014	—	0·0536
10	10	0·1015	—	0·0532
10	20	0·1030	—	0·1081
10	20	0·1025	—	0·1081
10	30	0·1028	—	0·1625
10	30	0·1025	—	0·1627
10	30	—	0·1030	0·1625

The table shows that when the amount of magnesium as oxide exceeds the amount of calcium as oxide a little of the former is precipitated with the latter metal. In this case the precipitate of calcium oxalate is ignited, redissolved in a little phosphoric acid and precipitated again. This has always given us correct results.

The ease with which calcium compounds dissolve in phosphoric acid might find application in several ways. For example we find that this acid enables us to make an excellent separation of calcium and barium sulphates; calcium sulphate readily dissolves but barium sulphate is quite insoluble in this acid.

Again calcium fluoride is soluble in the acid on heating. We have not examined this reaction quantitatively, but on testing the filtered solution with oxalic acid followed by ammonia abundance of typical calcium oxalate crystals were seen when the precipitate was examined under the microscope.

REFERENCES.

Andrewes (1915). *Report of the Medical Officer L.G.B.*, 1913–1914, 179.
Aron (1907). *Biochem. Zeitsch.*, **4**, 268.
McCrudden (1910). *J. Biol. Chem.*, **8**, 83.
—— (1911). *J. Biol. Chem.*, **10**, 187.

XXVI. EXPERIMENTS ON THE INTERACTION OF HYDROXY- AND AMINO-ACIDS WITH NITROPHENYLHYDRAZINE WITH SPECIAL REFERENCE TO THE FORMATION OF GLYOXAL DERIVATIVES.

By HENRY DRYSDALE DAKIN.

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(*Received May 26th, 1916.*)

Dudley and the writer [1913, 1914] have shown that when α-hydroxy-acids, such as glycollic, lactic, glyceric or mandelic acid, are digested in aqueous solution with *p*-nitrophenylhydrazine, small amounts of substances identical in properties with the bis-nitrophenylhydrazones of glyoxals are formed. Amino-acids, such as glycine, alanine, valine, leucine, aspartic acid and phenylalanine, in feebly acid solution under similar circumstances gave the same products. The course of the reaction was crudely represented as follows:

$$R \cdot CHOH \cdot COOH \rightleftarrows R \cdot CO \cdot CHO + H_2O$$
$$R \cdot CHNH_2 \cdot COOH \rightleftarrows R \cdot CO \cdot CHO + NH_3$$

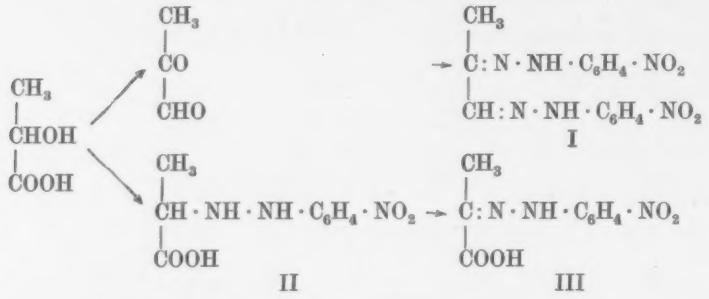
But it should be recalled that the direct synthesis, outside the body, of amino-acids from glyoxals by the action of ammonia or similar substances was never observed.

The qualitative demonstration of the reactions upon which these conclusions are based is, in some respects, easy to carry out, but the yield of glyoxal derivatives is extremely small and their bis-nitrophenylhydrazones are not readily converted into other derivatives and moreover the reactions are complicated by numerous side changes.

The object of the following paper is to record a few additional experiments which throw some light on the reaction. These experiments are manifestly incomplete but as the opportunity to carry them further is at the moment lacking, it appeared worth while to describe them now.

(I) *Formation of* p-*nitrophenylhydrazides from α-hydroxy-acids.*

It has already been shown that when dilute lactic acid is digested with *p*-nitrophenylhydrazine, the following products are formed—(i) methylglyoxal bis-nitrophenylhydrazone, (ii) α-nitrophenylhydrazinopropionic acid, (iii) pyruvic acid nitrophenylhydrazone. This last substance was derived from the hydrazino-acid (ii), from which it is readily obtained by oxidation.



The amount of these three products was however not nearly enough to account for the amount of nitrophenylhydrazine which disappeared. It has now been found that a fourth product, namely, the *p*-nitrophenylhydrazide of lactic acid, $CH_3 \cdot CHOH \cdot CO \cdot NH \cdot NH \cdot C_6H_4 \cdot NO_2$, is formed in relatively large amounts. The substance possesses attractive properties and is easily prepared. A similar crystalline derivative was prepared from *d*-lactic acid and from glycollic acid, while α-hydroxybutyric acid and β-hydroxybutyric acid gave only oily products.

Glycollyl-p-*nitrophenylhydrazide,* $CH_2OH \cdot CO \cdot NH \cdot NH \cdot C_6H_4NO_2$. Glycollic acid (2 g.), nitrophenylhydrazine (4 g.), water (20 cc.) and alcohol (20 cc.) were warmed on a water bath, then diluted with 100 cc. of water and allowed to stand overnight. Most of the unchanged nitrophenylhydrazine was precipitated by adding 2 cc. of saturated sodium acetate solution and the remainder was extracted from the filtrate by shaking with three successive portions of chloroform. The hydrazide was next separated by repeated extraction with amyl alcohol. The latter solvent was removed by evaporation at a low temperature under diminished pressure and the product (3·1 g.) was recrystallised from boiling alcohol in which it is only moderately soluble. The substance crystallises from alcohol in lemon yellow needles melting at 192°–194°. It is readily soluble in water, very sparingly soluble in chloroform or benzene and is easily hydrolysed on boiling with acids. On analysis the

substance gave 20·1 per cent. nitrogen compared with 19·9 for the calculated amount.

i-*Lactyl*-p-*nitrophenylhydrazide.* Lactic acid (8·5 g.), *p*-nitrophenylhydrazine (17 g.) and 95 per cent. alcohol (100 cc.) were heated on the water bath in a loosely corked flask for a couple of hours, a little more alcohol was then added and the mixture allowed to stand overnight in an incubator at 37°. Most of the alcohol had then evaporated and the mixture was diluted with half a litre of water. A small precipitate of unchanged base was filtered off and the rest was removed by three extractions with chloroform. The hydrazide was then extracted by repeated shaking with amyl alcohol. The extract was evaporated under diminished pressure and the solid residue of crude hydrazide (15 g.) was recrystallised from a mixture of alcohol and toluene. The yield was 12 g.

In another experiment the reaction was carried out in aqueous solution with excess of lactic acid. Nitrophenylhydrazine (2 g.) was heated for two hours on the water bath with lactic acid (5 cc.) and water (45 cc.). After standing overnight the solution was partly neutralised by adding sodium carbonate until a pink colour transitorily appeared and then successively extracted with chloroform and amyl alcohol as before. The solid crystalline residue was dissolved in alcohol (5 cc.) and toluene added to the warm solution to a point just short of the appearance of turbidity. A yield of 2·1 g. of the hydrazide was obtained.

Lactyl-*p*-nitrophenylhydrazide is an almost colourless substance when pure and crystallises from alcohol in prisms and plates which sinter at 162° and melt at 165°–167°. It is readily soluble in water, alcohol and acetone, sparingly soluble in ether, chloroform and toluene, insoluble in light petroleum. On analysis the substance gave 18·6 per cent. nitrogen compared with a calculated value of 18·7 per cent.

d-*Lactyl-nitrophenylhydrazide.* This substance was prepared from *d*-lactic acid in the same way as the inactive variety. Two g. of the acid and 3 g. of the hydrazine gave 2·1 g. of product crystallising from alcohol and toluene in fine prisms melting at 161°–163° with slight previous sintering. On analysis the substance gave 18·9 per cent. nitrogen. The substance is strongly laevorotatory. Its rotation was observed in absolute alcohol solution.

$$c = 2{\cdot}35, \qquad l = 2 \text{ dm}, \qquad \alpha = 1{\cdot}37°,$$

$$[\alpha]_D^{17°} = -34{\cdot}5°.$$

Another preparation had $[\alpha]_D^{17°} = -33{\cdot}6°$.

(II) *Conditions affecting the formation of methylglyoxal derivatives and other substances from lactic acid and nitrophenylhydrazine.*

It has been shown that when aqueous lactic acid is digested with *p*-nitrophenylhydrazine at least four well characterised substances are found in addition to indefinite decomposition products arising from the hydrazine. The results of some preliminary experiments relating to the formation of these compounds may be referred to. The products of the reaction were separated in a roughly quantitative fashion as follows. The mixture of aqueous lactic acid and nitrophenylhydrazine prepared as described in previous papers [1913, p. 130], after digestion at 37° for a determined period, was filtered through a weighed Gooch crucible. The insoluble part consists of methylglyoxal-bis-nitrophenylhydrazine with α-nitrophenylhydrazinopropionic acid and the nitrophenylhydrazone of pyruvic acid. The last two acid substances were separated by washing the precipitate with hot sodium carbonate solution and recovering them from the alkaline solution by acidifying with acetic acid. The very sparingly soluble methylglyoxal-bis-nitrophenylhydrazone was then washed with water and alcohol. The original aqueous solution after filtering off the precipitate was examined for unchanged hydrazine by acetone while lactyl-nitrophenylhydrazide was separated by partial neutralisation with sodium carbonate, half saturation with ammonium sulphate and extraction with amyl alcohol.

It does not seem of value to record a number of these experiments in detail but the following definite points were established.

(*a*) If the lactic acid is completely neutralised with sodium hydroxide before digestion with nitrophenylhydrazine no formation of methylglyoxal-bis-nitrophenylhydrazone is observed. A free carboxyl group is apparently necessary for methylglyoxal formation.

(*b*) Mineral acids added in small amounts (e.g. 2 per cent. sulphuric acid) to lactic acid solutions (10 per cent.) with nitrophenylhydrazine (0·5 per cent.) inhibit phenylhydrazide formation and slightly diminish the yield of methylglyoxal derivative. The yield of hydrazino-acid is not materially influenced.

(*c*) When aqueous lactic acid and nitrophenylhydrazine are digested together at 37°, a low concentration of lactic acid (e.g. 2 per cent.) favours both the formation of methylglyoxal-bis-nitrophenylhydrazone and of the hydrazino-acid but is unfavourable to the formation of lactyl-nitrophenylhydrazide. Concentrated solutions (e.g. 10 per cent. and over) favour hydrazide formation but are prejudicial to the production of the other products.

(*d*) Formation of methylglyoxal-bis-nitrophenylhydrazone is not conditioned by the prior formation of lactyl-nitrophenylhydrazide since the latter substance could not be converted into the former.

As an example of the relative yields of products the following experiment may be cited. Lactic acid (300 cc.), water (300 cc.) and *p*-nitrophenylhydrazine (30 g.) were rapidly heated to boiling, cooled and filtered. The mixture was divided into three parts; (*a*) was digested undiluted at 37°, the others, (*b*) and (*c*), were diluted with one and three litres of water respectively and digested for ten days. After three days practically all unchanged hydrazine had disappeared from (*a*) while a little more remained in (*b*) and (*c*). The yields of methylglyoxal-bis-nitrophenylhydrazine from (*a*), (*b*) and (*c*) were respectively 31, 77 and 203 milligrams. The yields of α-nitrophenylhydrazino-propionic acid were 116, 482 and 808 milligrams respectively. Almost the whole of the remaining nitrophenylhydrazine was converted into lactyl-nitrophenylhydrazide.

(III) *Glyoxal derivatives from other acids.*

As stated in the introduction, evidence of the formation of glyoxal derivatives has been observed in the case of a number of α-hydroxy- and α-amino-acids when digested with nitrophenylhydrazine. Since the yield of these glyoxal derivatives is very small, it appeared desirable to make comparative experiments with other acids, in order to see if confirmatory evidence could be obtained as to the character of the reaction under investigation.

The following experiments were simply of a qualitative kind. In order to detect glyoxal formation the precipitates obtained on digestion with nitrophenylhydrazine were filtered off and washed first with hot sodium carbonate solution and then with alcohol. The residue was then tested with caustic soda solution and alcohol. A clear blue colour resembling that of Fehling's solution is taken as a probable indication of glyoxal formation since all bis-nitrophenylhydrazones yield this extremely sensitive reaction. Brown, reddish or violet colourations such as many nitrophenylhydrazine derivatives yield are not obtained if the glyoxal derivatives are properly purified with sodium carbonate and alcohol.

The solutions were usually prepared by warming together 4 g. of the acid, 40 cc. of water and 0·2 g. nitrophenylhydrazine. The solutions were rapidly cooled, filtered and digested at 37°. After two or three days any precipitate was filtered off, and examined as described. The results were as follows:

Acid		Reaction of product with sodium hydroxide
Butyric acid	$CH_3 \cdot CH_2 \cdot CH_2 \cdot COOH$	–
α-Hydroxybutyric acid	$CH_3 \cdot CH_2 \cdot CHOH \cdot COOH$	+
β-Hydroxybutyric acid	$CH_3 \cdot CHOH \cdot CH_2 \cdot COOH$	–
Crotonic acid	$CH_3 \cdot CH=CH \cdot COOH$	?
Isobutyric acid	$(CH_3)_2 \cdot CH \cdot COOH$	–
α-Hydroxy-isobutyric acid	$(CH_3)_2 \cdot COH \cdot COOH$	+
α-Methoxy-isobutyric acid	$(CH_3)_2 \cdot C(OCH_3) \cdot COOH$	–
Diethylglycollic acid	$(C_2H_5)_2 \cdot COH \cdot COOH$	+
Tartaric acid	$COOH \cdot CHOH \cdot CHOH \cdot COOH$	+
Gluconic acid	$CH_2OH \cdot (CHOH)_4 \cdot COOH$	+

The product from crotonic acid gave a blue colour with caustic soda typical of glyoxal derivatives but the substance differed from the latter in being readily decomposed by hydrochloric acid. It appears likely that some substance with two adjacent nitrophenylhydrazine groups was formed but its structure is quite obscure.

(IV) *Action of semicarbazide on leucine.*

In all the experiments hitherto recorded on the formation of glyoxals from α-hydroxy- and α-amino-acids use has been made of nitrophenylhydrazine as being peculiarly suitable on account of the extraordinarily low solubility of its glyoxal derivatives. Dudley and the writer have shown that isobutylglyoxal gives a fairly insoluble compound with semicarbazide [1914, p. 38]. Accordingly an attempt was made to isolate isobutylglyoxal semicarbazone on long digestion of leucine with semicarbazide sulphate. Unfortunately the experiment gave a negative result. Whether this is due to a too great solubility of the semicarbazone or whether glyoxal formation is in some way conditioned by the presence of nitrophenylhydrazine is not clear. Further work is needed to determine these points. In any case it should be recalled that the simple equation given at the commencement of this paper to represent the dissociation of α-hydroxy- and α-amino-acids is adopted for convenience in explaining certain experimental results rather than on account of a belief in its precise accuracy. Many other alternative forms of dissociation are conceivable.

REFERENCES.

Dakin and Dudley (1913). *J. Biol. Chem.*, **15**, 127.
—— —— (1914). *J. Biol. Chem.*, **18**, 29.

XXVII. THE OXIDATION OF AMINO-ACIDS TO CYANIDES.

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(*Received May 31st, 1916.*)

The oxidation of α-amino-acids to aldehydes may be effected with a variety of reagents including hydrogen peroxide [Dakin, 1906], lead dioxide and dilute sulphuric acid [Liebig, 1849], alloxan [Strecker, 1862; Hurtley and Wootton, 1911], glyoxals [Dakin, 1914], and also with sodium hypochlorite [Langheld, 1909]. In all of these and many similar reactions, the nitrogen of amino-acids seems very easily removed by oxidation. The action of sodium hypochlorite on amino-acids was carefully studied by Langheld who showed that in all probability the first stage in the reaction was conditioned by the formation of a monochloroamino-acid, which subsequently underwent decomposition as follows:

$$R \cdot CH(NH_2) \cdot COOH \xrightarrow{+NaClO} R \cdot CH(NHCl) \cdot COONa \xrightarrow{+H_2O} R \cdot CHO + NH_3 + NaCl + CO_2.$$

In the case of leucine Langheld isolated the monochloroamino-acid as an unstable solid and obtained good evidence for the formation of a dichloroamino-acid when excess of hypochlorite was used. Apparently he did not investigate the decomposition products of the dichloroamino-acids.

Recently the writer has been engaged with the investigation of the properties of sulphochloroamides such as sodio-*p*-toluenesulphochloroamide, a substance prepared by the combination of sodium hypochlorite and toluene-sulphonamide, and now used to some extent as an antiseptic under the name chloramine-T. It has already been shown that though a solution of this substance contains no free hypochlorite, it reacts with α-amino-acids much in the same way as hypochlorite, yielding aldehydes, carbon dioxide

and ammonia [Dakin, Cohen, Daufresne and Kenyon, 1916]. The corresponding aldehydes were prepared from glycine, alanine, leucine, aminophenylacetic acid and methylaminophenylacetic acid.

Now sodio-*p*-toluenesulphochloroamide gives a practically neutral solution and the products of its decomposition are neutral. It appeared therefore that this substance might prove to be a useful neutral oxidising agent suitable for the oxidation of rather unstable substances. Langheld believed that he had obtained a small amount of glyoxaline acetaldehyde from histidine by oxidation with hypochlorite but the characterisation of the substance left much to be desired. It was decided, therefore, to try the action of sodio-*p*-toluenesulphochloroamide on histidine in the hope of obtaining more favourable results. A considerable yield of a crystalline product was obtained which was at first thought to be the aldehyde but which on investigation proved to be cyanomethyl-glyoxaline, a substance already prepared by Pyman [1911]. At first this appeared to be an anomalous result but it now appears that the reaction is of a fairly general character and that cyanides may be obtained by the oxidation of a variety of amino-acids. Apparently the cyanides are produced by the decomposition of previously formed dichloroamino-acids:

$$R \cdot CH(NH_2) \cdot COOH \rightarrow R \cdot CH(NCl_2) \cdot COOH \rightarrow R \cdot CN + 2HCl + CO_2.$$

On applying the reaction to other amino-acids, traces of hydrocyanic acid were obtained from glycine, acetonitrile from alanine, isobutyl cyanide (isovaleronitrile) from leucine and cyanobenzene (benzonitrile) from aminophenylacetic acid. The yields of cyanide in the case of the last three amino-acids were large.

As a matter of fact the reaction is not entirely novel, for Langheld apparently overlooked the fact that Schwanert [1857] many years ago before the constitution of leucine was settled had obtained clear proof of the formation of isobutyl cyanide when alkaline solutions of leucine were treated with chlorine. Liebig [1849] also stated that leucine on treatment with manganese dioxide and sulphuric acid gave isobutyl cyanide, while glycine gave hydrocyanic acid.

In this connection Plimmer's [1904, 1, 2] observation of the formation of hydrocyanic acid from glycine and other amino-acids on oxidation with nitric acid may be recalled; also the production of isobutyl cyanide from gelatin and caseinogen on oxidation with chromic acid recorded by Schlieper [1846] and Guckelberger [1847].

The oxidation of α-amino-acids to cyanides may have more than a theoretical interest in certain cases since it affords an additional method for the preparation of amines from amino-acids. Histidine for example may be oxidised to cyanomethyl-glyoxaline and then reduced to β-aminoethyl-glyoxaline without much difficulty and with relatively good yields.

Experimental.

Glycine and Alanine. The production of formaldehyde and acetaldehyde from glycine and alanine (1 mol) when their aqueous solutions are warmed with sodio-*p*-toluenesulphochloroamide (1 mol) has already been recorded [Dakin and colleagues, 1916]. When two molecular proportions of the sulphochloroamide are used to one of glycine and the mixture distilled, the distillate contains traces of hydrocyanic acid which are easily recognisable by the customary tests. In the case of alanine the first portion of the distillate contains oily drops of acetonitrile which subsequently dissolve as the distillate becomes more dilute. A trace of chloroamine, NH_2Cl, is also usually present and is easily recognised by its irritating odour. It disappears on standing owing to decomposition. The acetonitrile is readily shaken out with ether and gives a crystalline hydrobromide sparingly soluble in dry ether. On hydrolysis with sulphuric acid the formation of acetic acid can easily be recognised.

Leucine. The synthetic amino-acid (3·6 g.) was gently warmed with a solution of sodio-*p*-toluenesulphochloroamide (13 g.) in 100 cc. of water and the mixture was then distilled. The oily layer consisting largely of isobutyl cyanide was separated and shaken with sodium bisulphite solution to remove a little isovaleric aldehyde. The cyanide was then taken up in a little ether and distilled from a small distilling flask. 1·8 g. of isobutyl cyanide were obtained corresponding to a yield of about 70 per cent. of the theoretical amount. The cyanide was hydrolysed by boiling under a reflux condenser with excess of fifty per cent. sulphuric acid, the mixture diluted with water and the isovaleric acid recovered by distillation. It was converted into the silver salt which was analysed.

0·5944 g. gave 0·3081 g. silver = 51·8 per cent. Ag

$C_5H_9O_2Ag$ requires 51·7 „ „ „

α-Amino-phenylacetic acid. This acid was oxidised with two molecular proportions of the sulphochloroamide in the same fashion as in the preceding

experiment with leucine. The oily distillate consisting of cyanobenzene mixed with some benzaldehyde was dissolved in a little ether, shaken vigorously with sodium bisulphite to remove benzaldehyde and then the ether layer washed successively with sodium carbonate solution and water. On evaporation of the ether a 60 per cent. yield of crude cyanobenzene was obtained. Part of this was hydrolysed with sulphuric acid to benzoic acid, M.P. 120°, and another portion was converted into benzamide by cautious treatment with alkali. The most satisfactory identification was effected by dissolving 0·5 g. of the oil in 3 cc. absolute alcohol, then adding 3 cc. of concentrated ammonia and saturating the mixture with sulphuretted hydrogen. The solution was then heated in a sealed tube at 100° for an hour, the alcohol evaporated off and the solid residue of thiobenzamide crystallised from boiling water. The yield of thiobenzamide was practically quantitative as stated by Gabriel [1890] and the substance crystallised from water in long fine felted needles melting at 117°.

Histidine. The material for the experiments with histidine was kindly furnished by Dr Ewins. The dihydrochloride (1 g.) was dissolved in 20 cc. of water, neutralised with 0·5 g. of sodium carbonate, and 2·6 g. of sodio-toluenesulphochloroamide was then added. After standing for some time the solution was filtered to remove toluenesulphonamide and the filtrate shaken a few times with ether to remove the bulk of any dissolved sulphonamide. This procedure involves some loss of the nitrile but this can be recovered by washing the extracted sulphonamide with a little water. The main filtrate was then concentrated, preferably under reduced pressure, and before taking to complete dryness excess of dry sodium carbonate was added. The dry residue was then extracted with boiling ethyl acetate. It was found most convenient to do this in a Soxhlet apparatus after mixing the residue with plaster of Paris. The ethyl acetate extracts the cyanomethyl-glyoxaline almost exclusively and on evaporation of the solvent the substance readily crystallises, but it is usually somewhat pigmented As much as 0·4 g. of the cyanomethyl-glyoxaline can be obtained from 1 g. of histidine dihydrochloride corresponding to about eighty per cent. of the theoretical amount.

The cyanomethyl-glyoxaline was purified either by direct crystallisation from water, or from a mixture of methyl alcohol and toluene, or it was converted into the sparingly soluble picrate or finally it was sublimed at a temperature just below its melting point under the very low pressure obtained with the Gaede pump. However obtained the substance melts sharply at 138° and has all the properties described by Pyman [1911].

A mixed melting point with material kindly supplied by Dr Pyman showed no change.

Part of the substance was converted into the picrate which melted sharply at 167° after recrystallisation from water. It was analysed for picric acid by means of Busch's "nitron" method [1905].

0·6148 g. picrate gave 0·9891 g. precipitate = 68·1 per cent. picric acid

$C_5H_5N_3 \cdot C_6H_2(NO_2)_3OH$ requires 68·1 „ „ „

The cyanomethyl-glyoxaline gave the abnormal diazo-reaction and yielded a very soluble hydrochloride agreeing exactly with Pyman's description.

A portion of the cyanomethyl-glyoxaline was reduced to aminoethyl-glyoxaline with sodium and alcohol. The physiologically active base gave the characteristic dipicrate melting at 238°.

The above experiments were carried out in the Biochemical Laboratories of the Medical Research Committee under a grant from the Committee and I am indebted to Drs Dale and Barger for facilitating the work in every way possible.

REFERENCES.

Busch (1905). *Ber.*, **38**, 861.
Dakin (1906). *J. Biol. Chem.*, **1**, 171.
—— (1914). *J. Biol. Chem.*, **18**, 32.
Dakin, Cohen, Daufresne and Kenyon (1916). *Proc. Roy. Soc. B.*, **89**, 232.
Gabriel (1890). *Ber.*, **23**, 158.
Guckelberger (1847). *Annalen*, **64**, 76.
Hurtley and Wootton (1911). *J. Chem. Soc.*, **99**, 288.
Langheld (1909). *Ber.*, **42**, 392, 2360.
Liebig (1849). *Annalen*, **70**, 313.
Plimmer (1904, 1). *J. Physiol.*, **31**, 65.
—— (1904, 2). *J. Physiol.*, **32**, 51.
Pyman (1911). *J. Chem. Soc.*, **99**, 668.
Schlieper (1846). *Annalen*, **59**, 15.
Schwanert (1857). *Annalen*, **102**, 228.
Strecker (1862). *Annalen*, **123**, 363.